Universidade de Aveiro Departamento de Química
2011

David José Souto Patinha

Análise de compostos triterpénicos na casca de espécies de *Eucalyptus*

Analysis of triterpenic compounds in the bark of *Eucalyptus* species

Universidade de Aveiro Departamento de Química
2011

David José Souto Patinha

Análise de compostos triterpénicos na casca de espécies de *Eucalyptus*

Analysis of triterpenic compounds in the bark of *Eucalyptus* species

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química, realizada sob a orientação científica da Doutora Carmen Freire, Investigadora Auxiliar do CICECO da Universidade de Aveiro e do Doutor Juan José Villaverde Mella, Estagiário de Pós-Doutoramento do CICECO e do Departamento de Química da Universidade de Aveiro.

Apoio financeiro do projecto AFORE (CP-IP 228589-2) no âmbito do VII Quadro Comunitário de Apoio

Dedico este trabalho aos meus pais, irmã, namorada e amigos pelo incansável apoio.

o júri

presidente

Prof. Doutor Artur Manuel Soares da Silva
Professor Catedrático do Departamento de Química da Universidade de Aveiro

Doutora Paula Cristina de Oliveira Rodrigues Pinto
Investigadora Auxiliar do LSRE da Faculdade de Engenharia da Universidade do Porto

Doutora Carmen Sofia da Rocha Freire Barros
Investigadora Auxiliar do CICECO do Departamento de Química da Universidade de Aveiro

Doutor Juan José Villaverde Mella
Estagiário de Pós-Doutoramento do CICECO e do Departamento de Química da Universidade de Aveiro

agradecimentos

Quero agradecer à Dra. Carmen Freire e ao Dr. Juan Villaverde pela excelente orientação científica, dedicação, compreensão, incentivo e paciência ao longo de todo o tempo.

Um agradecimento especial ao Prof. Doutor Armando J. D. Silvestre pelo acompanhamento total e contínuo deste trabalho.

Agradeço ao Prof. Doutor Artur Silva pela realização e discussão dos espectros de ressonância magnética nuclear.

Agradeço em especial à Sónia Santos e ao Rui Domingues pelas dicas e ajuda ao longo do trabalho.

Agradeço a todos os colegas (nacionais e estrangeiros) dos laboratórios, aquário, bancada e mesa pelo bom ambiente vivido, pelas gomas, pelas pausas para café e pelos conselhos.

Agradeço a toda a minha família mas principalmente aos meus pais Armanda e António pelo apoio total durante toda a minha formação pessoal e académica.
Agradeço à minha irmã Daniela por tudo o que tem feito por mim até hoje em todos os aspectos.
Um carinho especial à Catarina que me acompanha em todos os momentos.

Agradeço aos meus amigos Ângelo S., B. Reis, Bruno C., Humberto C., Mickael S.,Nuno F., Pedro F., Tiago B., Tiago M. pelo companheirismo e presença constante.

palavras-chave

Eucalyptus nitens, *Eucalyptus grandis x globulus*, biorrefinaria, casca, extração Soxhlet, ácidos triterpénicos, 3-hidroxiolean-18-en-28-oato de metilo, GC-MS, RMN.

resumo

Portugal tem na indústria de pasta e papel um forte sector da sua economia. Estas indústrias geram anualmente grandes quantidades de resíduos, sendo a casca um dos principais. O fim destes resíduos é normalmente a queima em caldeiras de biomassa para produção de energia, mas com o surgir do conceito da biorrefinaria, estes resíduos podem ser aproveitados de maneira mais eficiente, nomeadamente através da extração prévia da fração lipofílica das cascas de eucalipto ricas em ácidos triterpénicos com elevado valor comercial e atividades biológicas interessantes. Assim, o presente trabalho tem como principal objetivo o estudo dos extratáveis lipofílicos presentes na casca da espécie *Eucalyptus nitens* e do híbrido *E. grandis x globulus*. A cromatografia gasosa acoplada com a espectrometria de massa (GC-MS) foi a técnica base usada na caracterização destes extratos.

A análise das amostras de casca externa de *E. nitens* revelou teores em triterpenóides superiores aos referidos na literatura para outras espécies estudadas anteriormente, em particular no que diz respeito aos ácidos triterpénicos com cerca de 22 g/kg de casca. No híbrido *E. grandis x globulus*, o 3-hidroxiolean-18-en-28-oato de metilo, ou morolato de metilo, um éster triterpénico derivado do ácido morólico, com uma estrutura do tipo oleanano foi identificado como componente maioritário (~3 g/kg de casca externa). Além da análise por GC-MS, este composto foi isolado por cromatografia preparativa e detalhadamente caracterizado por espectrometria de massa com ionização por electrospary (ESI-MS), RMN de ^{1}H e ^{13}C, HSQC, HMBC, DEPT e NOESY.

Foram ainda obtidos os extratos em MeOH:H_2O (50:50) das cascas de *E. nitens* e de *E. grandis x globulus,* nos quais foi avaliada a capacidade antioxidante e o teor de fenóis totais. O *E. nitens* (~49 g AGE/kg casca) apresenta valores de fenóis totais superiores aos encontrados no *E. globulus* (~38g AGE/kg casca) e *E. grandis x globulus* (~35g AGE/kg casca). Contudo, o híbrido apresenta maior capacidade antioxidante (~45 g AAE/kg casca) relativamente ao *E. nitens* (~34 g AAE/kg casca) e *E. globulus* (~20 g AAE/kg casca).

keywords

Eucalyptus nitens, Eucalyptus grandis x globulus, biorefinery, bark, Soxhlet extraction, triterpenic acids, methyl-3-hydroxyolean-18-en-28-oate, GC-MS, NMR.

abstract

The pulp and paper industry is a strong sector of the Portuguese economy. These industries generate annually considerable amounts of biomass residues, among which bark is one of the most abundant. These residues are currently burned in biomass boilers for energy production, but with the emergence of the biorefinery concept, these residues can be more efficiently exploited, for example through the pre-extraction of the lipophilic fraction of *Eucalyptus* barks, which are rich in high value and bioactive triterpenic acids. In this perspective, the main objective of the present work is to study the chemical composition of the lipophilic extracts of *Eucalyptus nitens* and of the hybrid *E. grandis x globulus* barks. Gas chromatography coupled with mass spectrometry (GC-MS) was the main analytical technique used in the characterization of these extracts.

The analysis of the *E. nitens* outer bark fractions showed higher levels of triterpenoids than those found in the literature for other previously studied species, especially in terms of triterpenic acids accounting for about 22 g/Kg of bark. In *E. grandis x globulus* bark extracts, methyl-3-hydroxyolean-18-en-28-oate, or methyl morolate, an ester derived from morolic acid, with an oleanane type triterpene skeleton was identified as the main component (~3 g/Kg of bark). In addition to the GC-MS analysis, this compound was isolated by preparative chromatography and characterized in detail by electrospray ionization mass spectrometry (ESI-MS), ^{1}H and ^{13}C NMR, HSQC, HMBC, DEPT and NOESY.

Extracts in MeOH:H_2O (50:50) from the two bark samples studied were also obtained and the antioxidant activity and total phenolic content were evaluated. *E. nitens* (~49 g GAE/kg of bark) shows higher values for phenolic content than those found in *E. globulus* (~38 g GAE/kg of bark) and *E. grandis x globulus* (~35 g GAE/Kg of bark). However, the hybrid *Eucalyptus* species has higher antioxidant activity (~45 g AAE/Kg of bark) than *E. nitens* (~34 g AAE/Kg of bark) and *E. globulus* (~20 g AAE/Kg of bark).

ABBREVIATIONS

HVLV	HIGH VALUE-LOW VOLUME
LVHV	LOW VALUE-HIGH VOLUME
WHC	WHOLE CROP BIOREFINERY
GR	GREEN BIOREFINERY
LCF	LIGNOCELLULOSIC FEEDSTOCK BIOREFINERY
HMF	5-HYDROXYMETHYLFURFURAL
PLA	POLYLACTIC ACID
GC-MS	GAS CHROMATOGRAPHY-MASS SPECTROMETRY
HHDP	HEXAHYDROXYDIPHENOYL
TMS	TRIMETHYLSILYL
BSTFA	N,O-BIS(TRIMETHYLSILYL)TRIFLUOROACETAMIDE
TMSCL	TRIMETHYLSILYLCHLOROSILANE
ESI-MS	ELECTRONSPRAY IONIZATION – MASS SPECTROMETRY
NMR	NUCLEAR MAGNETIC RESONANCE
COSY	CORRELATION SPECTROSCOPY
HSQC	HETERONUCLEAR SINGLE QUANTUM COHERENCE
HMBC	HETERONUCLEAR MULTIPLE BOND CORRELATION
TPC	TOTAL PHENOLIC CONTENT
DPPH	2,2-DIPHENYL-1-PICRYLHYDRAZYL
BHT	3,5-DI-TERT-4-BUTYLHYDROXYTOLUENE
DCM	DICHLOROMETHANE
IS	INTERNAL STANDARD
FA	FATTY ACIDS
LCAA	LONG CHAIN ALIPHATIC ALCOHOLS
ST	STEROLS
TT	TRITERPENES
NOESY	NUCLEAR OVERHAUSER EFFECT SPECTROSCOPY
DEPT	DISTORTIONLESS ENHANCEMENT BY POLARIZATION TRANSFER

CONTENTS

1. PREAMBLE 1

1.1 THE BIOREFINERY CONCEPT 2

1.1.1 BIOREFINERIES CLASSIFICATION 4

1.2 FOREST RESOURCES 5

1.3 BIOREFINERY IN PULP INDUSTRY 7

1.3.1. *EUCALYPTUS* BARK 9

1.4 OBJECTIVES OF THE PRESENT STUDY 10

2. EXTRACTIVES 11

2.1. ALIPHATIC COMPOUNDS 11

2.2. TERPENES AND TERPENOIDS 12

2.3. PHENOLIC COMPOUNDS 14

2.3.1. SIMPLE PHENOLS AND PHENOLIC ACIDS (C_6, C_6C_1 AND C_6C_3) 14

2.3.2. STILBENES ($C_6C_2C_6$) 15

2.3.3. FLAVONOIDS ($C_6C_3C_6$) 16

2.3.4. LIGNANS $(C_6C_3)_2$ 16

2.3.5. TANNINS 17

3. MATERIALS AND METHODS 19

3.1. BARK SAMPLES 19

3.1.1. EXTRACTION 19

3.2. ANALYSIS OF THE EXTRACTS 20

3.2.1. GAS CHROMATOGRAPHY – MASS SPECTROMETRY ANALYSIS 20

3.2.2. ISOLATION AND CHARACTERIZATION OF THE MOST ABUNDANT COMPOUND PRESENT IN THE OUTER BARK FRACTION OF *E. GRANDIS X GLOBULUS* 21

3.2.3. TOTAL PHENOLIC CONTENT 22

3.2.4. ANTIOXIDANT ACTIVITY 23

4. RESULTS AND DISCUSSION 25

4.1 LIPOPHILIC FRACTION ANALYSIS 25

4.1.1. CHARACTERIZATION OF TOTAL BARK LIPOPHILIC EXTRACTIVES 27

4.1.2. CHARACTERIZATION OF INNER BARK LIPOPHILIC EXTRACTIVES 29

4.1.3. CHARACTERIZATION OF OUTER BARK LIPOPHILIC EXTRACTIVES 35

4.2. CHARACTERIZATION OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE 42

4.2.1. ^{1}H NMR 44
4.2.2. ^{13}C NMR 45
4.2.3. 2D NMR Studies 47
4.3. Total phenolic content and Antioxidant activity 51
5. Conclusions 53
5.1 Future work 53
6. References 55

LIST OF FIGURES

FIGURE 1 - REPRESENTATION OF A BIO-BASED ECONOMY (ADAPTED FROM KAMM *ET AL* [3]). 1

FIGURE 2 – LIGNOCELLULOSIC BIOMASS COMPOSITION. 3

FIGURE 3 - SCHEMATIC DIAGRAM OF THE BIOREFINERY CONCEPT. 3

FIGURE 4 - PORTUGUESE FORESTED AREA, BY DOMINANT SPECIES, IN 2005/2006. 5

FIGURE 5 - REPRESENTATION OF DIFFERENT PARTS FROM *EUCALYPTUS GLOBULUS*, *E. NITENS* AND *E.GRANDIS*. 6

FIGURE 6 - CONCEPT OF AN INTEGRATED BIOREFINERY APPLIED IN A PULP AND PAPER INDUSTRY. 8

FIGURE 7 - STRUCTURES OF SOME COMMON ALIPHATIC COMPOUNDS. 11

FIGURE 8 - STRUCTURES OF MONO AND SESQUITERPENES IDENTIFIED IN *EUCALYPTUS GLOBULUS* [26]. 12

FIGURE 9 - EXAMPLES OF TRITERPENOIDS FOUND IN *E. GLOBULUS* BARK [14]. 13

FIGURE 10 - EXAMPLES OF TWO STEROLS FOUND IN WOOD AND HIGHER PLANTS. 14

FIGURE 11 - EXAMPLES OF SIMPLE PHENOLS, PHENOLIC ACIDS AND CINNAMIC ACIDS PRESENT IN PLANTS. 15

FIGURE 12 - EXAMPLES OF STILBENES FOUND IN PLANTS. 15

FIGURE 13 - EXAMPLES OF FLAVONOIDS, ISOFLAVONOIDS AND ANTHOCYANIDINS. 16

FIGURE 14 - EXAMPLES OF LIGNANS FOUND IN *EUCALYPTUS SPP*. 17

FIGURE 15 - STRUCTURE OF THE HIDROLYSABLE TANNIN BIS (HHDP)-GLUCOSE FOUND IN *E. GLOBULUS*. 18

FIGURE 16 - *EUCALYPTUS NITENS* BARK, A; *EUCALYPTUS GRANDIS X GLOBULUS* BARK, B; 2CM^2 EXTERNAL BARK, C; AND MILLED INTERNAL BARK, D. 19

FIGURE 17 - SOXHLET APPARATUS PARTS: FLASK (1), SOXHLET EXTRACTOR (2) AND CONDENSER (3). 20

FIGURE 18 – DCM EXTRACTION YIELDS FOR NON-MILLED BARK EXTRACTION AND A MILLED BARK EXTRACTION. 26

FIGURE 19 – GC-MS CHROMATOGRAMS OF THE DICHLOROMETHANE EXTRACTS OF *E. NITENS* (UPPER) AND *E. GRANDIS X GLOBULUS* (LOWER) TOTAL BARK WITH TETRACOSANE AS INTERNAL STANDARD (IS). FA-FATTY ACIDS, LCAA-LONG CHAIN ALIPHATIC ALCOHOLS, ST-STEROLS AND TT-TRITERPENES. 27

FIGURE 20 - TYPICAL GC-MS CHROMATOGRAMS OF THE DERIVATIZED DICHLOROMETHANE EXTRACTS OF *E. NITENS* (UPPER) AND *E. GRANDIS X GLOBULUS* (LOWER) INNER BARKS. 29

FIGURE 21 - MASS SPECTRUM OF THE TMS DERIVATIVE OF *B*-SITOSTEROL. 33

FIGURE 22 - MASS SPECTRUM OF THE TMS DERIVATIVE OF HEXADECANOIC ACID. 34

FIGURE 23 - MASS SPECTRA OF THE TMS DERIVATIVE OF OCTACOSAN-1-OL. 34

FIGURE 24 - TYPICAL GC-MS CHROMATOGRAMS OF THE DERIVATIZED DICHLOROMETHANE EXTRACTS OF *E. NITENS* (UPPER) AND *E. GRANDIS X GLOBULUS* (LOWER) OUTER BARKS. 35

FIGURE 25 - STRUCTURES OF TRITERPENOIDS IDENTIFIED IN *EUCALYPTUS NITENS* OUTER BARK. 37

FIGURE 26 - MASS SPECTRUM OF THE TMS DERIVATIVE OF URSOLIC ACID. 38

FIGURE 27 - MASS SPECTRA OF THE TMS DERIVATIVE OF BETULINIC ACID. 39

FIGURE 28 - MASS SPECTRUM OF THE TMS DERIVATIVE OF 3-ACETYLURSOLIC ACID. 39

FIGURE 29 - STRUCTURES OF TWO PHENOLIC COMPOUNDS IDENTIFIED IN THE OUTER BARK FRACTION OF 40

FIGURE 30 - MASS SPECTRUM OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE TMS DERIVATIVE. 42

FIGURE 31 – STRUCTURE OF MOROLIC ACID FOUND IN THE BARK OF *E. GROSSA*. 43

FIGURE 32 - MAIN CLEAVAGES AND RELATIVE ABUNDANCES OF TMS DERIVATIVE OF METHYL MOROLATE UNDER EI CONDITIONS. 44

FIGURE 33 - ^{1}H NMR SPECTRUM OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE. 45

FIGURE 34 - ^{13}C NMR SPECTRUM OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE. 46

FIGURE 35 - ALLYLIC CORRELATION PRESENT IN THE COSY SPECTRUM. 47

FIGURE 36 - COSY SPECTRUM OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE. 47

FIGURE 37 - HMBC CORRELATIONS OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE. 48

FIGURE 38 - HMBC CORRELATIONS OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE. 49

FIGURE 39 - HMBC CORRELATION OF METHYLIC PROTONS WITH CARBON FROM DOUBLE BOND. 49

FIGURE 40 - NOESY CORRELATION BETWEEN PROTON FROM HCOH WITH METHYLIC PROTONS FROM C_4. 50

LIST OF TABLES

TABLE 1 - BIOLOGICAL AND PHARMACEUTICAL PROPERTIES OF SOME COMPOUNDS IDENTIFIED IN EUCALYPTUS BARK. 10

TABLE 2 - LIPOPHILIC COMPONENTS (MG OF COMPOUND/KG OF DRY BARK) IDENTIFIED IN THE DICHLOROMETHANE EXTRACT OF THE INNER BARK OF *E. NITENS* AND *E. GRANDIS X GLOBULUS*. THE PEAK NUMBERS REFERS ONLY TO THE CHROMATOGRAMS ON THE FIGURE 20. 31

TABLE 3 - LIPOPHILIC COMPONENTS (MG OF COMPOUND/KG OF DRY BARK) IDENTIFIED IN THE DICHLOROMETHANE EXTRACT OF THE OUTER BARK OF *E. NITENS* AND *E. GRANDIS X GLOBULUS*. THE PEAK NUMBERS REFERS ONLY TO THE CHROMATOGRAM ON THE FIGURE 24. 36

TABLE 4 - MAJOR TRITERPENIC COMPOUNDS IDENTIFIED IN *EUCALYPTUS* SPECIES OUTER BARKS (G/KG OF DRY BARK). 41

TABLE 5 - MAJOR FAMILIES OF COMPOUNDS IDENTIFIED IN *EUCALYPTUS* SPECIES (MG/KG OF DRY BARK)..... 41

TABLE 6 - ^{1}H AND ^{13}C NMR CHEMICAL SHIFTS OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE IN $CDCL_3$ WITH TMS AS INTERNAL STANDARD..... 46

TABLE 7 – YIELD AND TOTAL PHENOLIC CONTENT OF THE TWO *EUCALYPTUS* SPECIES STUDIED. 51

TABLE 8 - ANTIOXIDANT ACTIVITY OF THE TWO BARK EXTRACTS STUDIED 51

1. PREAMBLE

Since 12000 years ago, humankind had agriculture as a source to meet most of their needs in terms of food but also of materials and energy, making possible the social, cultural and economic changes which shaped the modern world [1]. However, since the beginning of the twentieth century, the humankind development has been based primarily on the exploitation of fossil resources, leading to an exponential growth of the petrochemical industry, which has been the main supplier of energy, fuels and materials to the society. All this caused radical changes in the economy and living standards of developed countries. However, it is well known that this model of development is not sustainable. The main drawback of this system is the decrease of finite raw material reserves, which are being spent at a rate that cannot be maintained indefinitely. Other negative aspects are the overuse of these materials and the huge accumulation of non-biodegradable wastes. The oil-based products have reached their goals, but the secondary effects of their overuse are causing a massive impact on humans and biosphere in terms of greenhouse gas emissions and pollution, that needs to be stopped. Therefore, the global economy will have to transit from a model based on fossil resources to one based on renewable resources. In this context, the available biomass will have to be exploited in an efficient way to obtain materials and energy, leading to a low carbon footprint society and a new economic model based on bio-energy, bio-fuels and bio-based products as the main pillar of the so-called bio-economy (Figure 1) [2-4].

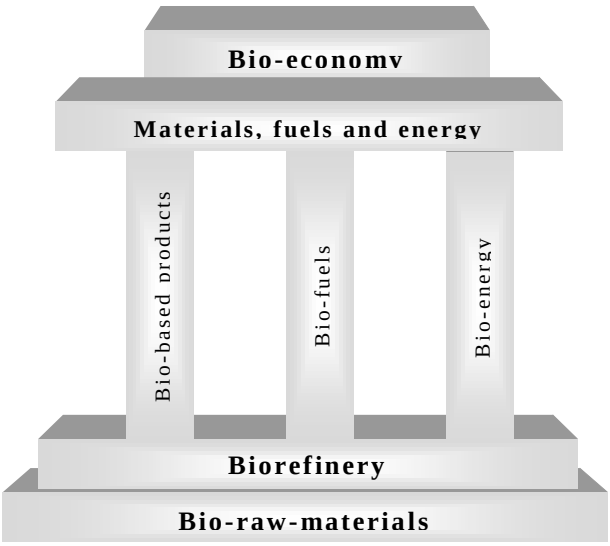


Figure 1 - Representation of a bio-based economy (adapted from Kamm *et al* [3]).

This new economic model is based on a new type of industrial exploitation called biorefinery. It uses biomass as raw material instead of fossil fuels used in existing refineries. However, this transition cannot be made without a deep reflection about some issues related to the utilization of the available biomass, paying particular attention in questions related with the right selection of plant species and their production increase. In addition, issues like the rational use of soils, the use of fertilizers and pesticides, the water consumption and the use of genetically modified organisms are also topics that have to be carefully analyzed.

Another point of special importance will be the analysis of how this production increase will affect the price and availability of food, ensuring that production meets the needs of an increasingly urban global population [5, 6]. Taking into account these concerns and other that may emerge in between, the implementation of a bio-economy still have a long way to go. Examples like the production of bioethanol and biodiesel are excellent starting points to the replacement of fossil resources. The implementation of biorefineries will certainly offer an enormous contribution in this new economic trend [6].

1.1 THE BIOREFINERY CONCEPT

As previously stated, a sustainable future will be based on proper and increasingly intelligent use of available biomass. The term “biomass” is often used for all biodegradable and non fossilized organic material which comes from plants, animals or micro-organisms [7-9]. Biomass has a quite complex and diverse chemical composition[10] (Figure 2) that requires a preliminary separation of its main components. Similarly to an oil refinery, where crude oil is fractionated to obtain diesel, gasoline, naphtha, kerosene, etc., also biomass undergoes a fractionation into its major components namely cellulose, hemicelluloses and lignin.

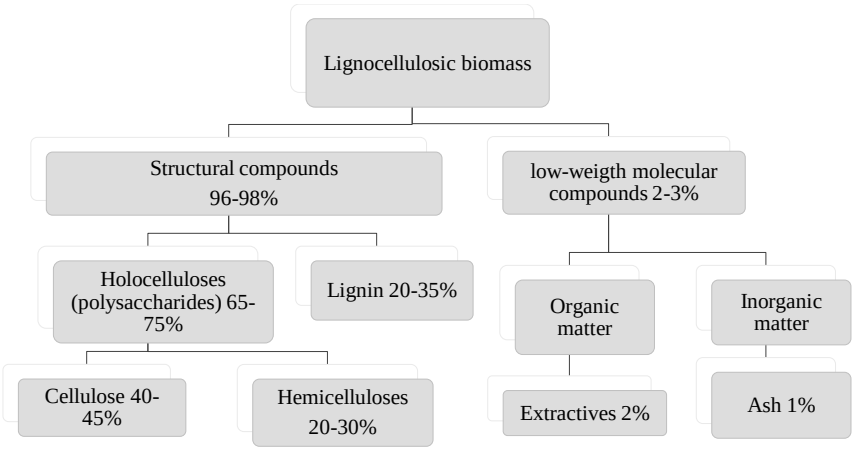


Figure 2 – Lignocellulosic biomass composition.

Figure 3 describes the conversion systems of a biorefinery. Through the production of multiple products, biorefineries can take advantage of the biomass complexity and maximize the value derived from this feedstock. The main goal is to co-produce high-value but low-volume products (HVLV) and low-value but high-volume (LVHV) products using multiple operations [11]. The operations should be designed to maximize the valued products and minimize the waste streams, generating electricity and process heat for its own use and perhaps enough for sale. Doing this, the high-value products enhance the profitability, while the high-volume fuels help to meet the global energy demand and the power production reduces costs avoiding greenhouse-gas emissions.

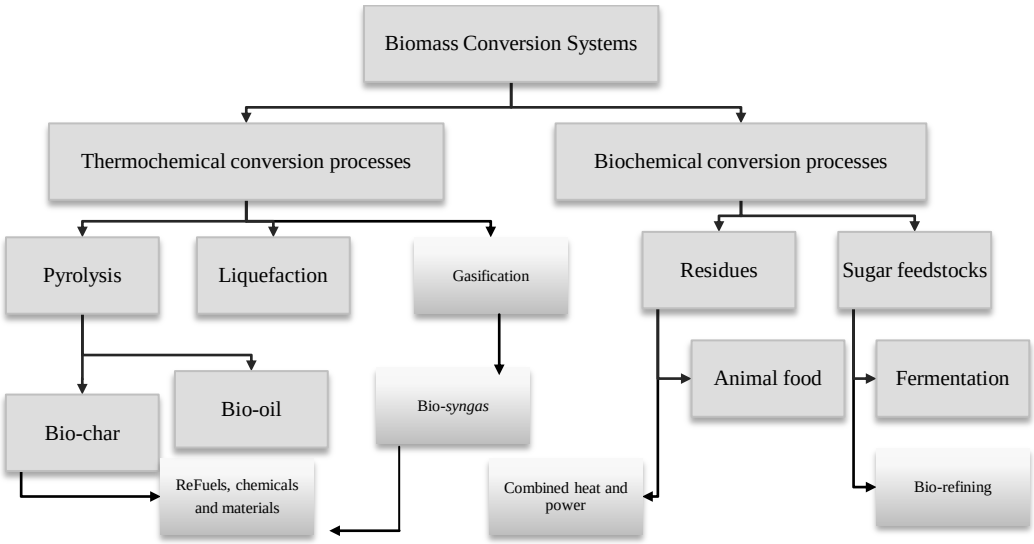


Figure 3 - Schematic diagram of the biorefinery concept.

1.1.1 BIOREFINERIES CLASSIFICATION

Biorefineries can be distinguished in three levels - phase I, II or III - according to their technological development, raw materials and processing technologies [3].

A phase I biorefinery is characterized by having a limited number of raw materials, with little flexibility of processes and limited products [12]. A dry mill ethanol plant is an example of a phase I biorefinery which produces a fixed amount of ethanol, other feed products and carbon dioxide, but with almost no processing flexibility [11].

Phase II biorefineries are similar to phase I, the difference here is the capability to produce various end products having far more processing flexibility, depending on market needs [3]. A system involving wet milling technology could be considered a phase II biorefinery. The typical products are starch, high fructose corn syrup, ethanol, and corn oil [11].

The phase III biorefineries are the most developed. In this case, it is possible to use a mixture of biomass feedstock producing a wide range of products, based on both the HVLV and LVHV products, by employing combination of technologies [3, 11, 12]. The Phase III biorefineries can be further sub-divided according to the raw material, namely: i) whole-crop biorefinery (WhC) – which processes and consumes the entire crop. Raw materials such as wheat, rye, triticale, and maize can be used as feedstock; ii) green biorefinery (Gr) - uses natural wet feedstock derived from untreated products, such as grass, green plants, or green crops and iii) lignocellulose feedstock biorefinery (LCF) - uses hard fibrous plant materials generated by lumber, forestry residues or municipal wastes. Plant materials are cleaned and broken down into the three main fractions (hemicelluloses, cellulose, and lignin) [11].

Another interesting concept is the integrated biorefinery that is an approach that optimizes the use of various types of biomass for the production of multiple materials and energy, minimizing waste and using a wide conversion technology platform which cuts across the various types of biorefineries [6, 13].

1.2 FOREST RESOURCES

The importance of the forest resources, as in the Portuguese economy, is well known. The pulp and paper industry, together with the cork industries, is one of the most important activities related with forest exploitation in Portugal, and it is mainly based on the use of *Eucalyptus globulus* tree (or tasmanian blue gum) [14, 15]. According to the 2009 report of the National Forest Authority, the Portuguese forest area occupies about 3.1×10^6 ha (Figure 4). Cork tree (*Quercus suber*), with 7.4×10^5 ha planted is the most common tree followed by pine tree (*Pinus pinaster)*, with 7.1×10^5 ha. In third place, with 6.5×10^5 ha of forested area, appears the *Eucalyptus*, mainly the *E. globulus* species [16].

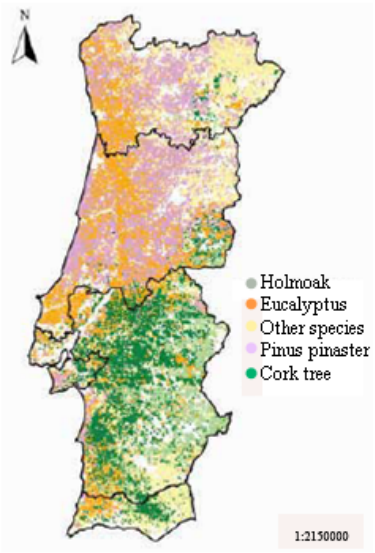


Figure 4 - Portuguese forested area, by dominant species, in 2005/2006.

E. globulus (Figure 5) is a rapid growing tree, that could attain about 14 m in the first 3 years and the possibility of reaching about 70 m high and 2 m in diameter, usually with a rough, greyish or brownish bark at the base, decorticating above in long strips leaving a smooth yellowish or greyish surface [17]. Due to the properties of the fibers obtained and the easy adaptation to the temperate climate [18] and soil, this species has become an important source of fast-growing pulpwood from plantations established in Australia, but also in Chile, Spain and Portugal [15, 17, 19], where it also helps small farmers with honey production.

In other countries, such as South Africa, Brazil, Uruguay, India and Zimbabwe, the most used species in the pulp and paper industry, is *Eucalyptus grandis* (or flooded gum) (Figure 5) certainly due to the rainfall limits which are 1000–3500 mm per year

compared to 600–1400 mm per year for *E. globulus*. *E. grandis* is a tall to very tall tree that grows up to 55 m high and reaches a maximum diameter of about 2 m. This subtropical tree [18] generally has 1–4 m of a short greyish rough bark at the base and above that, a smooth, powdery, white, greyish white or bluish grey bark [17].

Another *Eucalyptus* species that can be used in pulp and paper production is *Eucalyptus Nitens* [20] (or shining gum) (Figure 5). These are tall to very tall trees that can reach 70 m high and a maximum diameter of 2 m, with smooth and greyish bark, decorticating in long ribbons in the top and with a rough form at the base. These species inhabit high altitude slopes; having mountaintops as common habitats. During winter, *E. nitens* resist in areas where snowing is common [17, 21].

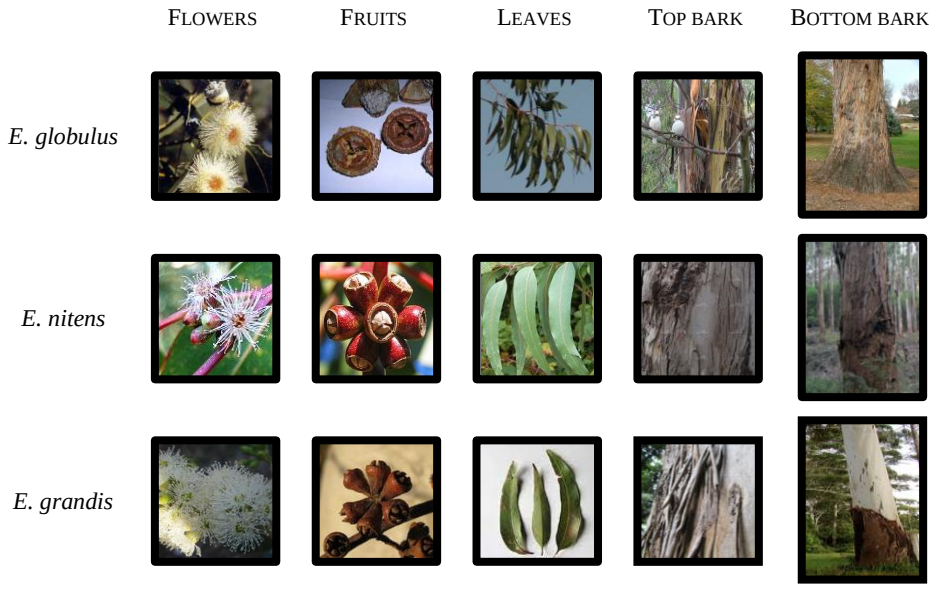


Figure 5 - Representation of different parts from *Eucalyptus globulus*, *E. nitens* and *E.grandis*.

In the past decades, biotechnology has been one of the sciences with higher developments and applications. Also in forestry, biotechnology has shown significant progresses. Thanks to new powerful biotechnological tools, potential advances in fields like the growth of more productive forests, healthier or better adapted to marginal conditions such as nutrient-poor soils, saline, water deficit, cold or bugs have been made essentially owed to hybridization techniques, leading to the creation of genetically modified organisms. Thereby, several hybrids of *Eucalyptus* species have already been obtained, such as *E. robusta* x *E. tereticornis*, *E. botryoides* x *E. tereticornis* or *E. globulus* x *E. nitens*. The hybrid between *E. grandis* and *E. globulus* is another example of a wide cross among eucalypt tree species and its bark composition will be discussed further in this thesis.

1.3 BIOREFINERY IN PULP INDUSTRY

Among the potential large-scale industrial biorefineries, the LCF biorefinery will be probably those with higher success [3] mainly because of the low cost of raw material such as straw, reed, grass, wood, paper-waste, corn stover, etc. [6, 8, 22]. Also, at an early stage, LCF biorefineries could be developed based on infrastructures of lignocellulosic materials industries such as pulp industries. These industries have already implemented systems of transport, storage and handling, among other requirements, that otherwise have to be built from zero. This will avoid the intensive initial investments, focusing on an early stage, not only in the production of energy in biomass boilers that is already made, but also on the exploitation of by-products like extractives, lignin or hemicelluloses from the production processes, without interfering with the main objectives of such industries. In the future, depending on markets demands, processing conditions can be adapted to maximize the production of other products and energy/fuels and to integrate alternative applications for the main products, as cellulose fibers [3].

Figure 6 shows a general outline of the operations of an integrated biorefinery in a pulp industry. A pre-extraction of hemicelluloses from wood prior to cooking or from black liquors (from which lignin is also isolated), as well as the separation of short cellulose fibers from long fibers after cooking are procedures that may add value to the wood material [23]. Hemicelluloses can be then used to produce ethanol, furfural or xylitol, while the long / short fibers separated after cooking, can be converted into ethanol, 5-hydroxymethylfurfural (HMF) or sorbitol [3, 24]. Note that, at an early stage of implementation, the cellulose fibers will only be used in the regular production of pulp and paper, not interfering with the main objective of this industry, but in the future, the exploitation of this resource can be redesigned to obtain other fibrous materials instead of paper, such as biocomposites, materials formed by a naturally occurring resin matrix or biodegradable synthetic polymer such as polylactic acid (PLA), reinforced with natural fibers of cellulose, making them more environmentally friendly and with various industrial applications [25].

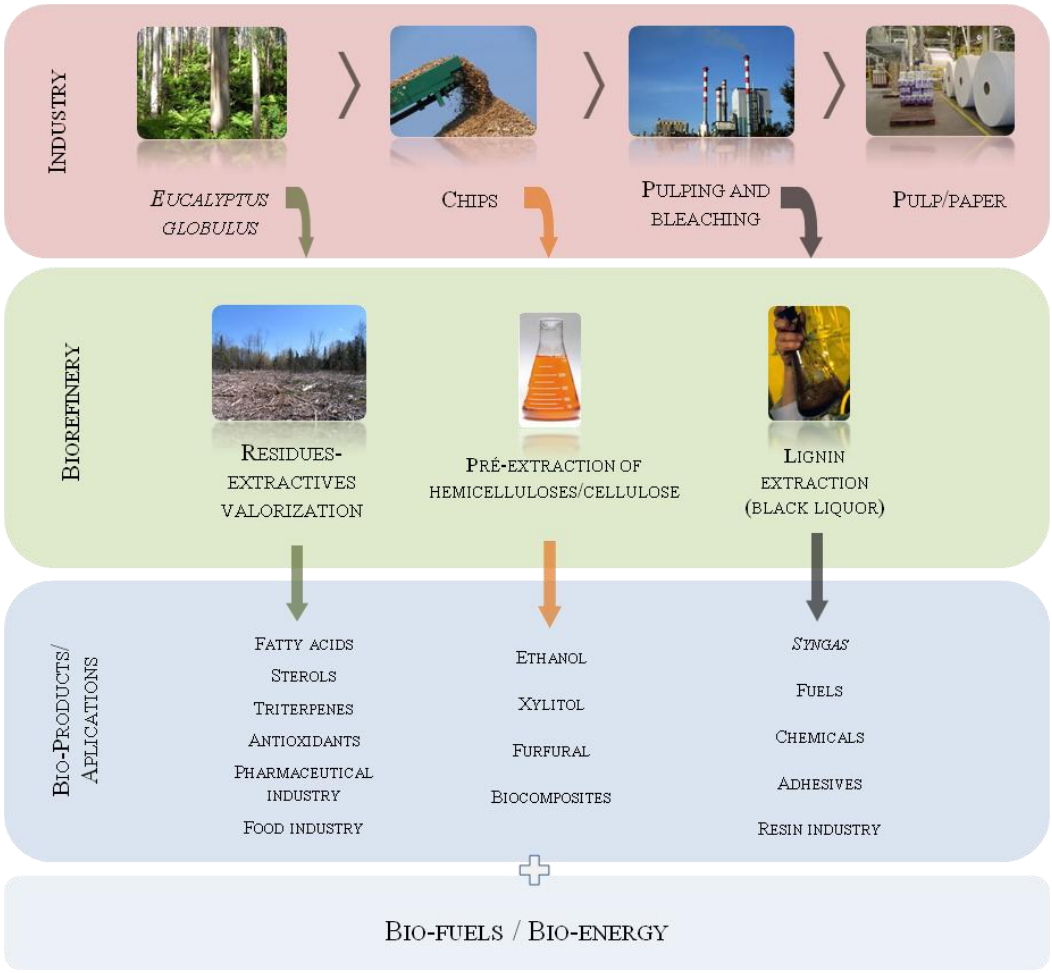


Figure 6 - Concept of an integrated biorefinery applied in a pulp and paper industry.

In addition to the cellulose fibers and hemicelluloses, lignin dissolved in black liquor offers a huge opportunity for the development of LCF biorefineries. Residual lignin from wood pulping is mainly burned for heat and power. However, it can find other added-value applications, that could be divided in two groups: insoluble lignin and chemically modified lignin [3].

The insoluble lignin belong to the LVHV applications such as cement additives or roads tar filling[3]; while the chemically modified lignin has a much wider variety of potential markets, like surfactants, detergents or biocides. One example is the depolymerisation of lignin by a base-catalyzed treatment obtaining a series of low molecular weight phenolic compounds [6, 11]. This mixture can be also subjected to hydroprocessing, which primarily yields a mixture of alkylbenzenes useful as potential liquid biofuels [6]. Another strategy is the thermal-cracking of lignin, using temperatures from 250 to 600 ˚C, that generates low molecular weight feedstock for further processing [6].

1.3.1. *EUCALYPTUS* BARK

A pulp mill with a production capacity of about 5.0×10^5 ton/year of bleached pulp can generate around 1.0×10^5 ton/year of bark [14]. These bark, among other residues like leaves or fruits are nowadays simply burned in biomass boilers for energy production or are simply left in the forest for soil nutrition [14]. In 2009, according to the Statistical Bulletin of CELPA (Paper Industry Association), the production of virgin pulp stood at 2.031 million tons that is equivalent to about 400 ktons of bark produced in Portugal in one year. These numbers, together with the chemical composition of this fraction alerts for the enormous interest on the up-grading of this unexploited renewable resource.

Freire *et al.* [26] reported that lipophilic extractives from *E. globulus* account for more than 10% of dry outer bark. This fraction revealed the presence of high commercial value compounds like sterols and triterpenoids with lupane, ursane and oleanane skeletons. These compounds have a wide range of activities (e.g. anti-tumoral effect, anti-HIV properties and cardiovascular protection, among other) that make them extremely valuable for biomedical applications (Table 1).

Among the triterpenic compounds found in *Eucalyptus globulus*, oleanolic, betulinic and ursolic acid, with contents around 4.0, 4.2 and 7.8 g/kg, respectively, could represent something like 21 ktons of ursolic acid per year. With an estimated commercial value in the range of 1295-1590 €/kg for 98% of purity, these values could be a push forward to the implementation of an integrated biorefinery, installed in a pulp mill.

Table 1 - Biological and pharmaceutical properties of some compounds identified in eucalyptus bark.

	a.Ca	a.Tu	a.Ul	a.Inf	a.Vir	a.Ma	a.HIV	a.Py	Ttr	a.Dia	Cht.	Nep	Car.p
Ursolic acid [27-30]	√	√	√	√	√								
Oleanolic acid [27, 28, 31]		√	√	√									
Betulinic acid [32-35]		√				√	√						
β-sitosterol [36-40]		√	√	√				√	√	√	√		
Resveratrol [41-44]	√	√		√	√					√		√	√
Taxifolin [45, 46]	√	√											
Matairesinol [47, 48]	√												√

(Anticarcinogenic (a.Ca), Antitumoral (a.Tu), Antiulcer (a.Ul), Anti-inflamatory (a.Inf), Antiviral (a.Vir), Antimalarial (a.Ma), Anti-HIV (a.HIV), Antipyretic (a.Py), Tuberculosis Treatment (Ttr), Antidiabetic (a.Dia), Cholesterol Treatment (Cht.), Neurological Protection (Nep.) and Cardiovascular Protection (Car.p))

1.4 OBJECTIVES OF THE PRESENT STUDY

The searching for a sustainable future will pass through the exploitation of the residues. The pulp and paper industry generates considerable amounts of residues which are not exploited to their full potential. In this context, this work evaluates the chemical composition of bark residues from two *Eucalyptus* namely *E. nitens* and *E. grandis x globulus*. These species have shown potential in the pulp and paper industries and for that, the characterization of the lipophilic extractives is important to evaluate their potential as sources of bioactive compounds, such as triterpenic acids. The main objectives were:

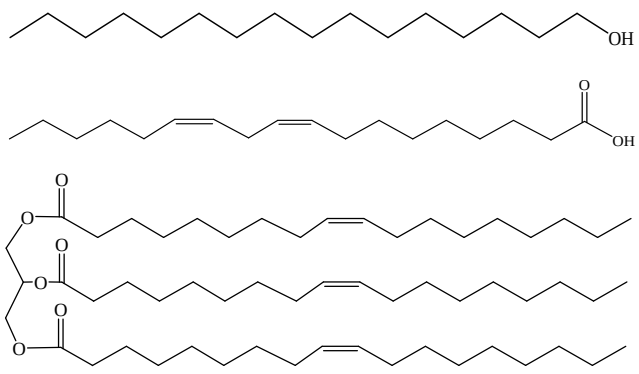
- To carry out the chemical characterization of the lipophilic extractives of *Eucalyptus nitens* and *Eucalyptus grandis x globulus* bark by GC-MS (Gas Chromatography-Mass Spectrometry)*;*
- To isolate the main unidentified compounds by GC-MS and characterize them by other techniques;
- To evaluate the antioxidant activity and total phenolic content of the polar extractives of bark.

2. EXTRACTIVES

"Extractives" is a term used to designate a variety of chemical components that are removable from biomass with various organic solvents. The chemical composition and amounts of this fraction varies with the species and also with factors like geographical location, time of the year, age, location on the tree, harvesting, transportation and storage [49-51]. This group of compounds act in trees as protection against parasites, water and contaminants barrier and in the wood colour definition [50]. Regarding some structural characteristics, extractives can be divided into three main groups: aliphatic compounds, terpenes and terpenoids and phenolic compounds [49].

2.1. ALIPHATIC COMPOUNDS

The aliphatic compounds found in vegetal biomass include alkanes, alcohols and fatty acids, both free and esterified [49]. Esters of aliphatic alcohols and fatty acids are know as waxes and are normally found in small amounts, whereas other esters of fatty acids are more abundant, mainly glyceryl esters in the form of mono-, di- or triglycerides (Figure 7). Steryl esters are also found showing several applications in food industry (e.g. margarine and yoghurts with cholesterol-lowering properties). On the other hand, free fatty acids and aliphatic alcohols, either saturated or unsaturated, are also present in this fraction. Aliphatic alcohols such as hexadecane-1-ol (Figure 7), octadecan-1-ol or tetracosan-1-ol and fatty acids such as palmitic, oleic or linoleic acids (Figure 7) were found in wood and bark extractives of *E. globulus* [52, 53].



Hexadecan-1-ol

Linoleic acid
(18:2 (9,12))

Triolein

Figure 7 - Structures of some common aliphatic compounds.

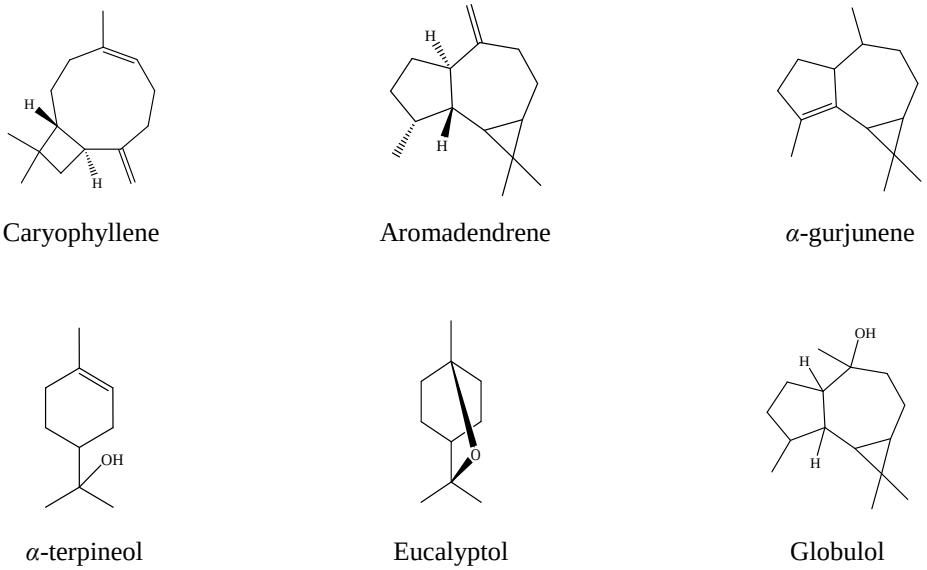
2.2. TERPENES AND TERPENOIDS

Terpenes are a large group of natural compounds derived from isoprene (2-methylbutadiene). Given the number of isoprene units that build these compounds, they are denominated as monoterpenes (2 units of isoprene), sesquiterpenes (3 units), diterpenes (4 units), sesterterpenes (5 units), triterpenes (6 units), tretraterpenes (8 units) and polyterpenes (> 8 units).

Monoterpenes (Figure 8) can be divided in acyclic, monocyclic and dicyclic compounds. As well as sesquiterpenes (Figure 8), they are mostly present in the volatile oils of certain morphological parts of plants, as in the leaves. Some of these compounds are often responsible for characteristics odours and others are known to have insect repellent properties [49, 54, 55]. In *E. globulus* some of the identified monoterpenes are eucalyptol and α-terpineol and several sesquiterpenes, namely α-gurjunene, aromadendrene, *allo*-aromadendrene, caryophyllene and globulol [26].

Diterpenes have a structure with 20 carbon atoms and they can be grouped into acyclic, monocyclic, dicyclic and tricyclic structural types [49, 56], being the last one the most common structure found in the oleoresin of softwoods [49, 51].

Triterpenes are composed of 30 carbon atoms and the most common structures have tetracyclic and pentacyclic arrangements.



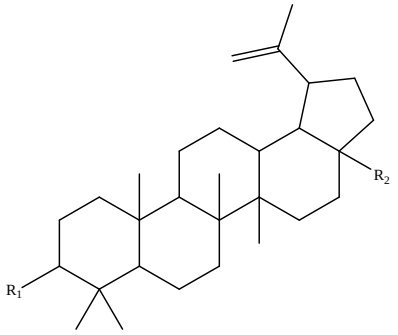
Caryophyllene | Aromadendrene | *α*-gurjunene

α-terpineol | Eucalyptol | Globulol

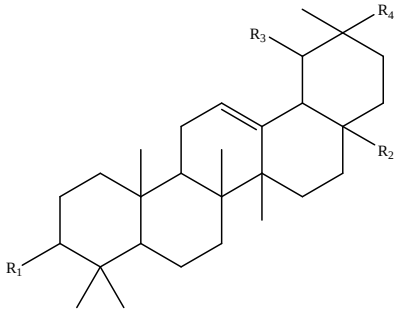
Figure 8 - Structures of mono and sesquiterpenes identified in *Eucalyptus globulus* [26].

Terpenes with one or more oxygen-containing functional groups such as alcohols, aldehydes, ketones and acids are known as **terpenoids** [49, 54]. In this class of compounds, triterpenoids are a large and structurally diverse group of natural products. They are derived from squalene or related acyclic 30-carbon precursors. Most triterpenoids are fused 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles, however, acyclic, monocyclic, bicyclic, tricyclic, and hexacyclic triterpenoids have also been isolated from natural sources [57].

Most of the triterpenic alcohols and acids found in the *Eucalyptus globulus* bark have a pentacyclic ring system with a hydroxyl group at C_3, specifically with ursane (e.g. ursolic acid), oleanane (e.g. oleanolic acid) and lupane type skeletons (e.g. betulinic acid) [14, 51, 56] (Figure 9).



R_1= OH; R_2= CH_3 Lupeol
R_1= OH; R_2= COOH Betulinic acid
R_1= OC(O)CH_3; R_2= COOH 3-Acetylbetulinic acid
R_1= =O; R_2= COOH Betulonic acid

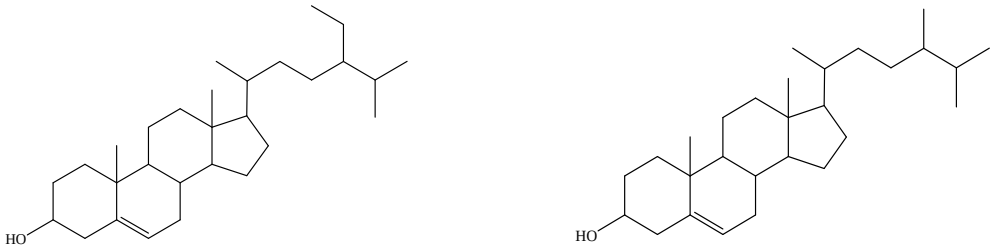


R_1= OH; R_2= CH_3; R_3= H; R_4= CH_3 β - Amyrin
R_1= OH; R_2= CH_3; R_3= CH_3; R_4= H α - Amyrin
R_1= OH; R_2= COOH; R_3= H; R_4= CH_3 Oleanolic acid
R_1= OC(O)CH_3; R_2= COOH; R_3= H; R_4= CH_3 3-Acetyloleanolic acid
R_1= OH; R_2= COOH; R_3= CH_3; R_4= H Ursolic acid
R_1= OC(O) CH_3; R_2= COOH; R_3= CH_3; R_4= H 3-Acetylursolic acid

Figure 9 - Examples of triterpenoids found in *E. globulus* bark [14].

Sterols are compounds closely related to triterpenoids, which have about 27 to 30 carbon atoms and a tetracyclic ring system, specific to steroids [54]. Normally, plant sterols (phytosterols) have a hydroxyl group at the C_3 position and a side chain with variable length at the C_{17} position. Double bonds and methyl groups are also found in these compounds being sometimes called methyl or dimethyl sterols due to the saturation of C_4. β-sitosterol is the most common phytosterol found in wood and higher

plants (Figure 10). Campesterol (Figure 10) is structurally similar, but it is found in fewer amounts than *β*-sistosterol. Besides their free form, sterols are also found as fatty acids esters (steryl esters) and as glycosides [49].



β-sitosterol Campesterol

Figure 10 - Examples of two sterols found in wood and higher plants.

2.3. PHENOLIC COMPOUNDS

These family includes compounds that have aromatic rings with hydroxyl groups in the structure [58]. Some of them contribute to the coloration of wood [59] and most of them have important biological properties [49]. The phenolic extractives could be sub-divided in simple phenols and phenolic acids, stilbenes, lignans, flavonoids and tannins.

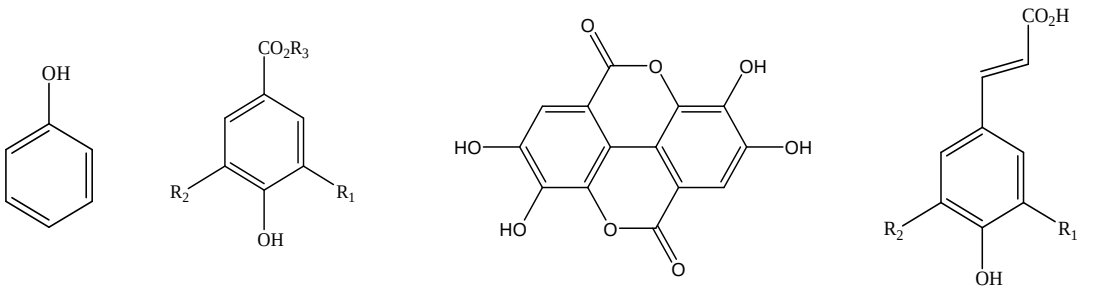
2.3.1. SIMPLE PHENOLS AND PHENOLIC ACIDS (C_6, C_6C_1 AND C_6C_3)

Phenol (Figure 11) is the simplest compound of this family. It has been identified in large quantities in wood of *Pinus silvestris* and *Picea abies* [59].

Benzoic type acids (C_6C_1), such as vanillic and syringic acids, seem to be widely present in plants, although in low concentrations and some aldehydes like vanillin and syringaldehyde were identified in wood of Norway spruce (*Picea abies*)[54]. The acids are found mainly covalently linked with other structural components in plants [60]. For example, gallic acid (Figure 11) and its dimmeric derivative, ellagic acid, are present mainly esterified as hydrolysable tannins as will be referred below. Santos *et al.*[61] identified some phenolic acids in *E. globulus* bark such as quinic acid, dihydroxyphenylacetic and methyl gallate.

The cinnamic type acids (C_6C_3) are the most frequent phenolic compounds and are found in the esterified and etherified form [62, 63]. Caffeic, *p*-coumaric, ferulic and sinapic acids are the most common elements of this family (Figure 11). Ferulic acid, for

example, is found in the free form in *Pinus radiata* [56], esterified in *Eucalyptus globulus* wood [19] and caffeic acid was found free in *E. globulus* bark [61].



Phenol

Vanillic acid - R_1=H R_2=OMe R_3=H
Gallic acid - R_1=OH R_2=OH R_3=H
Methyl gallate - R_1=OH R_2=OH R_3=Me

Ellagic acid

p-Coumaric acid - R_1=R_2=H
Ferulic acid - R_1=H R_2=OMe
Caffeic acid - R_1=H R_2=OH
Sinapic acid - R_1=R_2=OMe

Figure 11 - Examples of simple phenols, phenolic acids and cinnamic acids present in plants.

2.3.2. STILBENES ($C_6C_2C_6$)

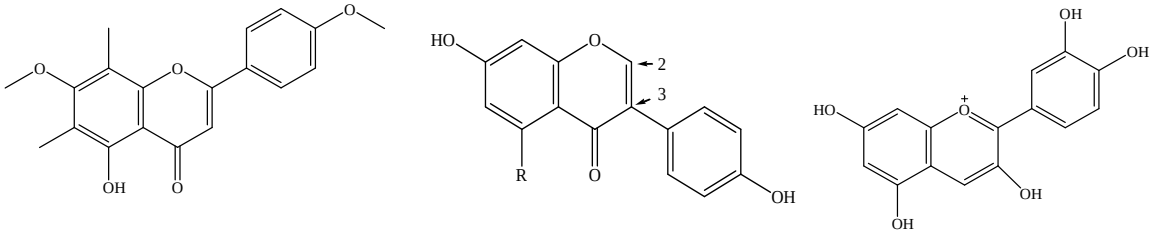
This is a small group of plant phenolic compounds found in wood, bark and leaves of both softwoods and hardwoods [56]; derived from 1,2–diphenylethylene, these compounds possess a conjugated double bond system [49]. Two examples of these compounds are pinosylvin and resveratrol (Figure 12). The first one is found in *Pinus* species and causes problems in the delignification process and in the colour reversion of pulps [54]. The second one (Figure 12) is another well know stilbene with cancer preventative activities, as evidenced in tissue culture and animal model studies [64] and has been identified in *E. sideroxylon* wood [65].

Pinosylvin Resveratrol

Figure 12 - Examples of stilbenes found in plants.

2.3.3. FLAVONOIDS ($C_6C_3C_6$)

Among vegetal polyphenols, flavonoids are the largest family, with thousands of structural variants described [66]. They are known to occur widely in woody plants and are found in both hardwoods and softwoods [56]. Flavonoids have a $C_6C_3C_6$ carbon skeleton and can be divided in various subclasses according to the degree of oxidation of the heterocyclic oxygen [58, 62]. Examples of these compounds are anthocyanines. They belong to a subclass of flavonoids responsible for the intense orange, red or blue colours of fruits, flowers, leaves and roots. Cyanidin and malvidin are two examples of anthocyanidines. The first one causes the reddish-orange colour of many red berries [67] while malvidin, found in *Vitis vinifera* is, is the responsible of the red wine colour [68]. In a recent work, Santos *et al.* [61] identified several flavonoids in the bark of *E. globulus* such as catechin, mearnsetin, quercetin, isorhamnetin, luteolin, taxifolin, eriodictyol, naringenin and several flavonoid glycosides such as isorhamentin-hexoside, quercetin-hexoside, myricetin-rhamnoside, aromadendrin-rhamnoside, phloridzin and mearnsetin-hexoside. Others found in *E. globulus* wood and bark are apigenin, eucalyptin (Figure 13) and sideroxylin, [69, 70]. The change from position 2 to the position 3 of the lateral aromatic ring linking gives origin to isoflavonoids (Figure 13). The most commons are daidzein and genistein (Figure 13), found in *Glycine max* [71].



Eucalyptin

Genistein - R = OH

Daidzein - R = H

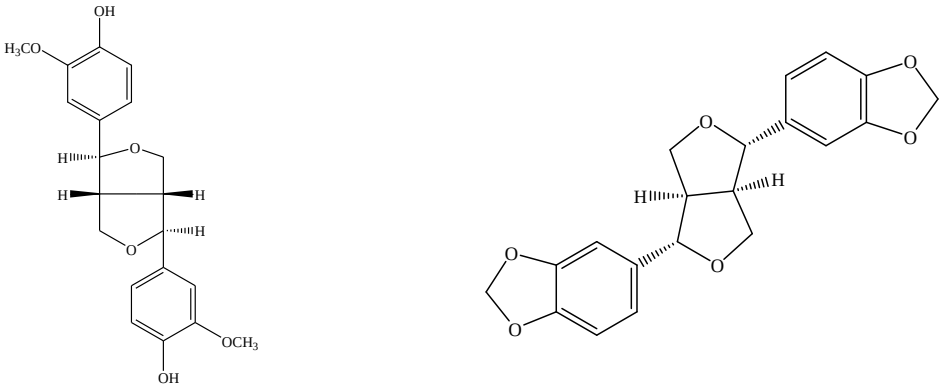
Cyanidin

Figure 13 - Examples of flavonoids, isoflavonoids and anthocyanidins.

2.3.4. LIGNANS $(C_6C_3)_2$

Lignans are a heterogeneous group of secondary plant metabolites formed mainly by oxidative coupling of two phenylpropane (C_6C_3) units, which may be bridged

with oxygen linkages [49, 56]. Some lignans have been suggested to be associated with low risk of cancer and in the treatment of HIV [72, 73]. Pinoresinol (Figure 14), globoidnan A and sesamin (Figure 14) are some lignans identified in *Eucalyptus spp* [72, 74, 75].



Pinoresinol Sesamin

Figure 14 - Examples of lignans found in *Eucalyptus spp.*

2.3.5. TANNINS

Tannins are an abundant family of oligomeric or polymeric compounds which can be divided in two groups:

i) hydrolysable tannins are composed by a monosaccharide, usually glucose, esterified with gallic acid (gallotannins) or ellagic acid (ellagitannins). Pedunculagin found in *E. nitens* wood [76] or eucalbanin and tellimagrandin I found in *E. globulus* [77], are examples of these type of compounds. Santos *et al* [61] also identified in *E. globulus* bark the bis(hexahydroxydiphenoyl(HHDP))-glucose (Figure 15) and galloyl-bis(HHDP)-glucose.

ii) condensed tannins are formed by polycondensation of the flavonoids catequin and leucoanthocyanidin and are therefore more resistant to chemical breakdown than hydrolysable tannins [49]. Condensed tannins of leucoanthocyanidin type were detected in *E. globulus*, *E. camaldulensis* and *E. rudis* wood and bark [78, 79].

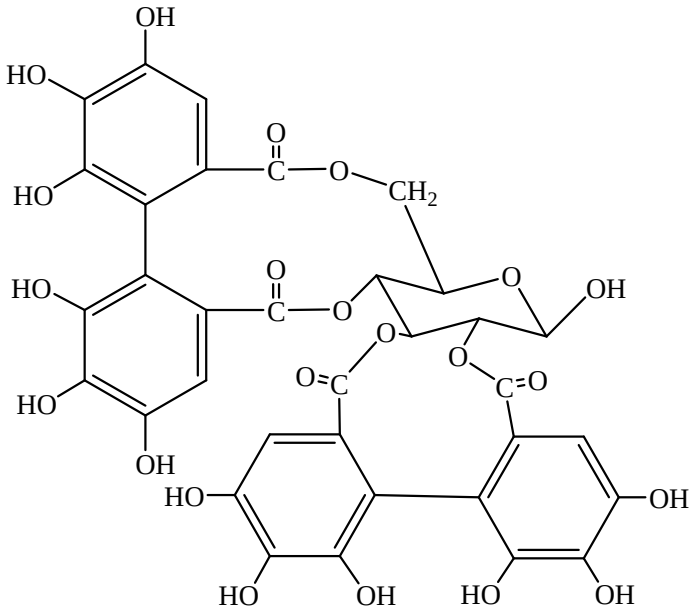


Figure 15 - Structure of the hidrolysable tannin bis (HHDP)-glucose found in *E. globulus*.

3. MATERIALS AND METHODS

3.1. BARK SAMPLES

The *Eucalyptus grandis x globulus* bark samples (Figure 16,b) used in this study were collected from 12 years old trees that came from two different plantations in Portugal, located in the north and in the south of the country. The bark samples of *E. nitens* (Figure 16,a) were collected from a 10 years old plantation and located on the outskirts of Águeda, north of Portugal.

The bark samples (total bark) were air dried until a constant weight was achieved. Then, their superficial layer (≈2mm) was removed and designated as outer bark. The remaining bark was denominated as the inner bark fraction. Some of the outer bark was cut into pieces of about $2cm^2$ (Figure 16,c) and the remaining outer, inner and total barks were milled in a Retsch SK1 cutting mill to granulometry lower than 2mm (Figure 16,d), prior to extraction.

a b c d

Figure 16 - *Eucalyptus nitens* bark, a; *Eucalyptus grandis x globulus* bark, b; $2cm^2$ external bark, c; and milled internal bark, d.

3.1.1. EXTRACTION

Extractives analysis typically includes extraction followed by analysis of component groups and individual compounds [51]. Solid samples, like bark and wood, are normally Soxhlet extracted (Figure 17) [80]. In this study, about 20g of bark were extracted with dichloromethane for 7h. Dichloromethane was chosen because it is a fairly specific solvent for lipophilic extractives [14]. The solvent was evaporated to

dryness, the extracts were weighted and the results were expressed in terms of dry biomass material. Further, the samples were characterized by GC-MS [14, 15, 19, 26, 81, 82].

Figure 17 - Soxhlet apparatus parts: flask (1), Soxhlet extractor (2) and condenser (3).

To carry out the quantification of the phenolic fraction, the total bark residue was also submitted to extraction with a methanol/water ($MeOH/H_2O$) mixture (50:50 (v/v)), at room temperature, for 24 h and under constant stirring. Then, the suspension was filtered, MeOH removed by low-pressure evaporation and the extract freeze-dried in a VirTis® benchtop K equipment.

3.2. Analysis of the extracts

3.2.1. Gas Chromatography – Mass Spectrometry analysis

Prior to GC–MS analysis, about 20 mg of each dried extract were trimethylsilylated according to the literature [14, 15, 26, 81-83]. The extracts, together with 0.25-0.50 mg of tetracosane (99% pure, Sigma) used as internal standard, were dissolved in 250 µL of pyridine. Compounds with hydroxyl and carboxyl groups were then converted into trimethylsilyl ethers and esters (TMS) respectively, by adding 250 µL of N,O-Bis (trimethylsilyl)trifluoroacetamide (BSTFA) and 50 µL of trimethylchlorosilane (TMSCl) and keeping the reaction mixture in an oil bath at 70 °C for 30 min. The GC–MS analysis was performed on a trace Gas Chromatograph 2000 series, equipped with a Thermo Scientific DSQII single-quadrupole mass spectrometer. Analysis of samples were carried out using a DB-1 J&W capillary column (30m × 0.32

mm inner diameter, 0.25 μm film thickness) using Helium as carrier gas (35 cm s^{-1}). The chromatographic conditions were as follows: initial temperature, 80 C for 5 min; temperature gradient, 4 °C/min up to 260 ºC; and 2 °C/min till the final temperature of 285 ºC and kept at 285 ºC for 10 min. Injector temperature, 250 °C; transfer-line temperature, 290 °C; split ratio, 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan s^{-1} over a range of *m/z* 33–750. The ion source was maintained at 250 °C.

Chromatographic peaks were identified on the basis of the comparison of their mass spectra with the equipment mass spectral library (Wiley-NIST Mass Spectral Library 1999), their characteristic retention times obtained under the described experimental conditions, and their fragmentation profiles with published work data. For quantitative analysis, GC–MS was calibrated with pure reference compounds, representative of the major lipophilic extractive components (namely, palmitic acid, 1-nonacosanol, *β*-sitosterol, betulinic acid, ursolic acid and oleanolic acid), relative to tetracosane, the internal standard. The respective response factors were calculated as an average of six GC–MS runs. Two aliquots of each extract were analyzed. Each aliquot was injected in triplicate. The presented results are the average of the concordant values obtained for each part (less than 5% variation between injections of the same aliquot and between aliquots of the same sample).

3.2.2. ISOLATION AND CHARACTERIZATION OF THE MOST ABUNDANT COMPOUND PRESENT IN THE OUTER BARK FRACTION OF *E. GRANDIS X GLOBULUS*

To carry out the isolation of the most abundant compound detected in the *E. grandis x globulus* outer bark and whose identification was not achieved based on the GC-MS analysis, 50 g of this outer bark were Soxhlet extracted with dichloromethane for 7 hours. The solvent was removed and the extract dried for gravimetric quantification.

The dried extract was redissolved in hexane, transferred to a separating funnel and washed with a potassium hydroxide (0.1 M) solution to exclude compounds such as fatty acids or triterpenic acids. The organic fraction was collected, concentrated and finally fractionated by column chromatography on silica gel, eluting with a mixture of ethyl acetate: petroleum ether. Several fractions were collected and analysed by GC-

MS. The fractions enriched in the desired compound were further fractionated by thin layer chromatography in silica gel, eluting with the same eluent used in the column chromatography, allowing to isolate the pure compound.

Electronspray Ionization – Mass Spectrometry (ESI-MS) and Nuclear Magnetic resonance (NMR) techniques (^{1}H, ^{13}C and 2D NMR) were then used to characterize the compound.

The ESI-MS and ESI-MS/MS analyses of the isolated compound were carried out in a QqQ Quattro (Micromass) using MassLynx software (version 4.0). MS spectra were obtained at least in triplicate, in the positive mode and the collision energy used was optimized to reduce the relative abundances of precursor ions of approximately 15%, varying the normalised collision energy between 15 and 40 eV.

Samples were prepared for analyses by dissolving the compounds with 200 μL of $CHCl_3$. Then 50 μL of this sample were diluted with 50 μL of methanol and introduced into the electrospray source at a flow rate of 0.6 mL min^{-1}, setting the needle and cone voltage at 30 V, ion source at 80 ºC and desolvation temperature at 150 ºC. Tandem mass spectra of the molecular ions were obtained by collision induced dissociation (CID), using argon as collision gas. The gas pressure in the Q2 collision cell was approximately $3.85x10^{-4}$ mbar. Full scan mass spectra, ranging from *m/z* 100 to 1000, and each spectrum, were produced by accumulating data during 1 minute [84].

NMR spectra were recorded on a Bruker DRX 300 spectrometer (300.13 and 75.47 MHz, for ^{1}H and ^{13}C, respectively), using $CDCl_3$ as solvent and TMS as internal reference. Unequivocal ^{1}H and ^{13}C assignments were made with the aid of 2 Dimensional (2D) Correlation Spectroscopy (COSY) ($^{1}H/^{1}H$), Heteronuclear Single Quantum Coherence (HSQC) ($^{1}H/^{13}C$) and Heteronuclear Multiple Bond Correlation (HMBC) experiments. The low-pass *J*-filter portion of the experiment was optimised for an average of one-bond heteronuclear coupling of 145 Hz; the delay for evolution of long-range couplings was optimised for 7 and 2 Hz.

3.2.3. TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) of the total bark extracts was determined by the Folin-Ciocalteu method [61, 85, 86]. 2.5 mL of Folin-Ciocalteu reagent, previously diluted

with water (1:10, v/v) and 2 mL of aqueous sodium carbonate (75 g/L), were added to accurately weighed aliquots of the total bark extract dissolved in 0.5 mL of water, corresponding to concentration ranges between 35 and 500 µg of extract/mL. Each mixture was kept for 5 min at 50 °C. After cooling, the absorbance was measured at 760 nm, using a Jasco® UV-vis V-530 spectrophotometer. The TPC was calculated as gallic acid equivalents from the calibration curve of gallic acid standard solutions (1.5 – 60 µg/mL) and expressed as mg of gallic acid equivalent (GAE)/g of dry extract. The analyses were carried out using three aliquots of extract, measured in triplicate and the final result was calculated as an average value.

3.2.4. ANTIOXIDANT ACTIVITY

The antioxidant activity of the total bark extract was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging [85, 87]. In test tubes, 0.25 mL of DPPH (0.8mM) in MeOH was added to 1 mL of the aqueous solution of the extract and 2.75 mL of MeOH, corresponding to concentrations of extract between 2.5 – 10.0 µg/mL. After mixing, the samples were maintained in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a Jasco® UV/Vis V- 530 spectrophotometer and compared with a control without extract. A blank was prepared using methanol instead of the DPPH solution. Ascorbic acid and 3,5-di-tert-4-butylhydroxytoluene (BHT) were used as reference compounds. Antioxidant activity was expressed as a percent inhibition of DPPH radical and calculated from the equation:

$$\text{Scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample}) \div \text{Abs control}] \times 100$$

IC50 values were determined from the plotted graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in µg/mL. Triplicate measurements were carried out. The antioxidant activity was also expressed in g of ascorbic acid equivalents/kg of dry bark (g AAE/kg dry bark).

4. RESULTS AND DISCUSSION

The main aim of this thesis was to study the chemical composition of the lipophilic extractives of *E. nitens* and *E. grandis x globulus* bark.

Preliminary studies of the effect of the size of the bark samples on the extraction yields and the determination of total phenolic content and antioxidant activity of the extracts were also carried out. The results obtained are presented in the next paragraphs, with more focus on the GC-MS analysis and characterization of the lipophilic fraction.

4.1 LIPOPHILIC FRACTION ANALYSIS

In order to study the effect of particle size on the extraction yield, some bark samples were milled and sieved to obtain particles of 2mm, while the remaining bark was cut into $2cm^2$ pieces. Due to the hardness of the inner bark, it was only possible to perform the extraction with the milled bark.

According to the results presented in Figure 18, the extraction yield increased, as expected, with the decrease of the particle size. The DCM extraction yield was significantly higher for both species when the milled bark was used, with 3.3% against 2.9% for *E. nitens* and 2.1% against 1.3 for *E. grandis x globulus* in the case of external bark. There are several well known explanations for this fact, such as the higher surface area of the samples and the easiest diffusion of the solvent in smaller particles [88].

The yields of the DCM extracts differ between the two *Eucalyptus spp.* studied and within the same species among the two morphological parts of bark (inner and outer bark). It was observed that the outer bark fractions are richer in lipophilic extractives than the inner bark. These results are in close agreement with those reported for *E. globulus* or *E. maidenii* [14, 26, 89].

The yields of the outer bark extracts of both species studied (2.1% for *E. grandis x globulus* and 3.3% for *E. nitens*) are in the range of values reported for other *Eucalyptus* species (e.g. 1.3% in *E. grandis*, 3.9% in *E. globulus* and 6.1% in *E.maidenii*) [26, 90]. The inner bark extract yields of *E. grandis x globulus* and *E. nitens* account for 0.23% and 0.32%, respectively. These values are also in the same range of those found for other *Eucalyptus* species studied before [14, 26, 89], but with *E. grandis x*

globulus inner bark as the fraction with lower extractive contents. The dichloromethane extractive yields of the total bark samples account for 0.68% for *E. nitens* and 0.69% for *E. grandis x globulus*. These values are more closed to those detected for the inner bark fractions due to the higher proportion of this morphological part.

These results also indicate that the outer barks of the most common species planted in temperate and Mediterranean zones, such as *E. globulus* and *E. nitens*, are richer in lipophilic extractives than the species of sub-tropical and tropical zones, such as *E. grandis* and *E. urograndis* [26, 89].

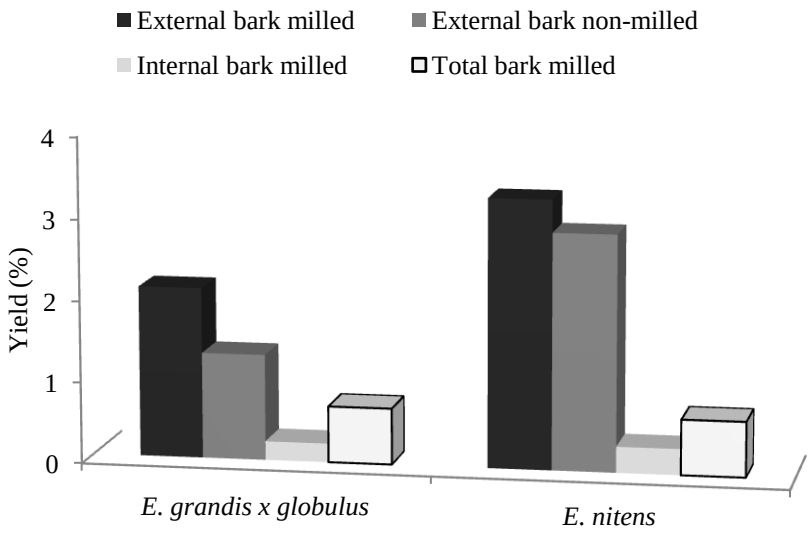


Figure 18 – DCM extraction yields for non-milled bark extraction and a milled bark extraction.

4.1.1. CHARACTERIZATION OF TOTAL BARK LIPOPHILIC EXTRACTIVES

A typical GC-MS chromatogram of the derivatized dichloromethane extract of *E. nitens* and *E. grandis x globulus* total bark are presented in Figure 19.

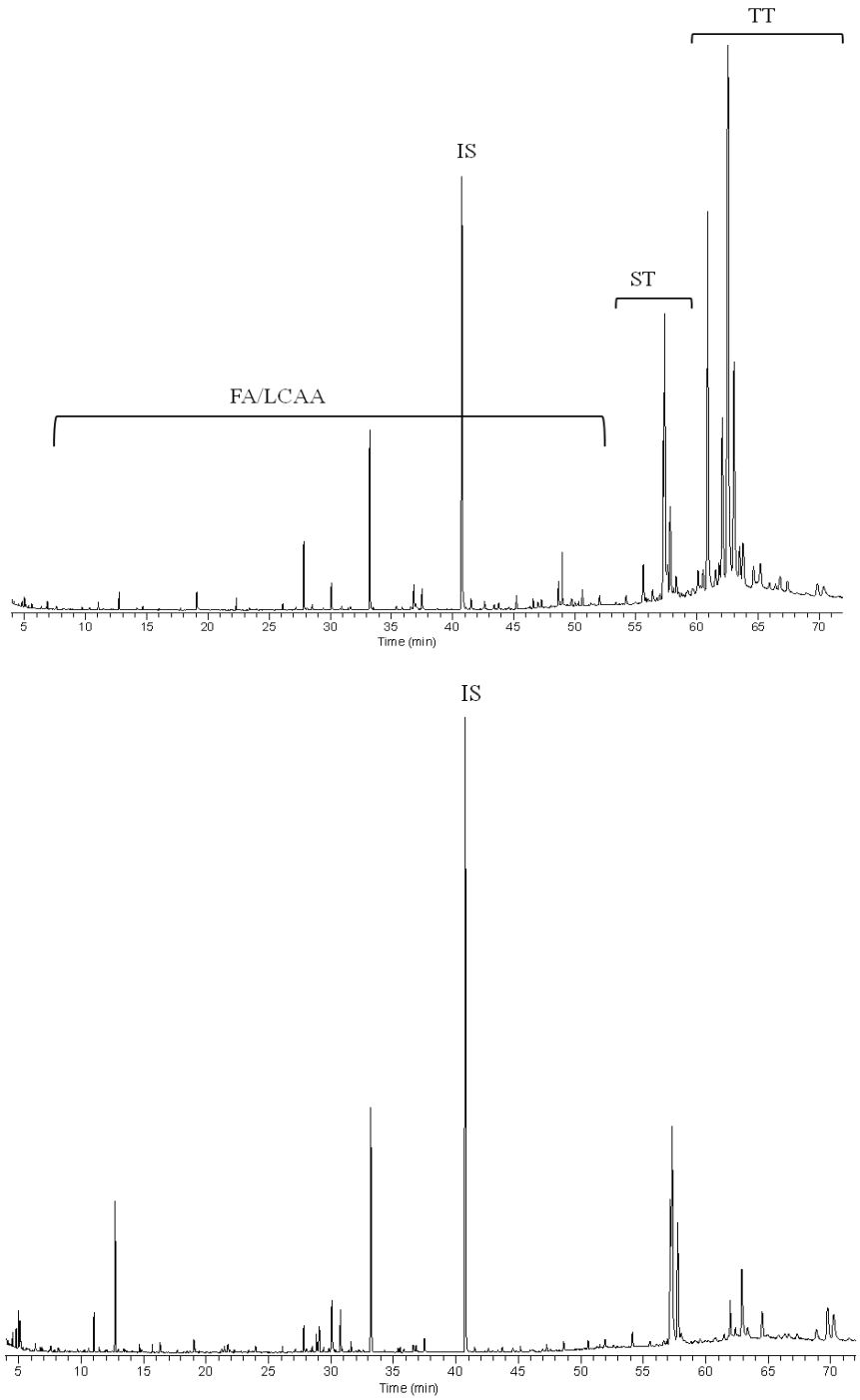


Figure 19 – GC-MS chromatograms of the dichloromethane extracts of *E. nitens* (upper) and *E. grandis x globulus* (lower) total bark with tetracosane as internal standard (IS). FA-Fatty acids, LCAA-Long chain aliphatic alcohols, ST-Sterols and TT-Triterpenes.

The GC-MS chromatograms show that fatty acids (FA), long chain aliphatic alcohols (LCAA), sterols (ST) and triterpenoids (TT) are the main families of compounds found in these extracts. Triterpenoids are the major components of the total bark extract of both species, with betulonic and betulinic acids as the main components found in *E. nitens* and *β*-amyrin in *E. grandis x globulus. β*-sitosterol is the most abundant sterol, while teracosan-1-ol, hexacosan-1-ol, octacosan-1-ol, oleic acid and linoleic acid are the most representative of long chain aliphatic alcohols and fatty acids respectively. Obviously, the composition of the total bark extractives reflect the relative composition of the two morphological fractions as will be discussed below.

4.1.2. CHARACTERIZATION OF INNER BARK LIPOPHILIC EXTRACTIVES

Figure 20 shows the typical GC-MS chromatograms obtained for the derivatized dichloromethane extracts of *E. nitens* and *E. grandis x globulus* inner barks and in Table 2 are compiled the lipophilic components (mg of compound/kg of dry bark) identified in these extracts.

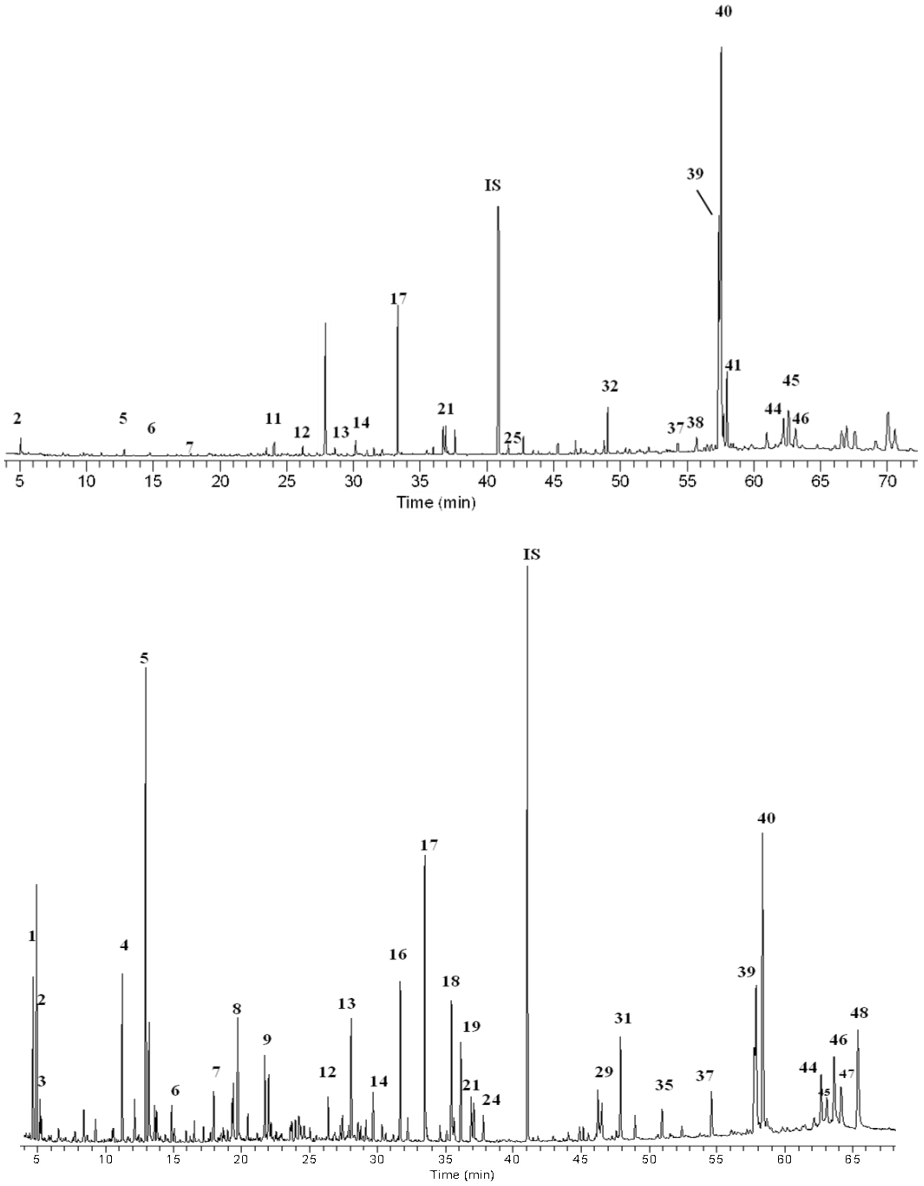


Figure 20 - Typical GC-MS chromatograms of the derivatized dichloromethane extracts of *E. nitens* (upper) and *E. grandis x globulus* (lower) inner barks.

Sterols and triterpenoids are the major compounds found in the *E. nitens* inner bark extract, with *β*-sitosterol **(40)** and *β*-amyrin **(39)** being the most abundant components and account for 0.5 and 0.3 g/kg of dry bark, respectively (Table 2) (numbers in bold refer to chromatographic peaks in the Figure 20). Triterpenic acids, such as oleanolic **(44)**, betulinic **(45)** and ursolic acids **(46)**, with a total content of 245.9 mg/kg were identified in lower quantities, but, as will be discussed later, represent the most predominant compounds found in outer bark fractions.

Some fatty acids such as linoleic **(21)** (29.7 mg/kg) and octadecanoic acid **(24)** (21.7 mg/kg) and long chain aliphatic alcohols like hexadecan-1-ol **(16)** (5.4 mg/kg) or octacosan-1-ol **(37)** (14.5 mg/kg) were also found in this extract.

In the dichloromethane extract of *E. grandis x globulus* inner bark, long chain aliphatic alcohols are the most abundant family of compounds, with a total amount of 205.8 mg/kg bark. Hexadecan-1-ol **(16)** (32.2 mg/kg), octadecen-1-ol **(18)** (51.1 mg/kg) and octadecan-1-ol **(20)** (24.9 mg/kg) are the most representative compounds of this family.

Fatty acids account for 132.4 mg/kg bark, with hexadecanoic **(17)** (53.2 mg/kg), heptadecanoic **(19)** (10.4 mg/kg) and tetracosanoic acids **(31)** (4.7 mg/kg) as the main compounds of this family.

However, although fatty acids and long chain aliphatic alcohols are the major families, the most abundant compound is *β*-sitosterol (82.4 mg/kg). This sterol seems to be specific of the inner bark extracts as well as the low content of triterpenic acids.

Table 2 - Lipophilic components (mg of compound/kg of dry bark) identified in the dichloromethane extract of the inner bark of *E. nitens* and *E. grandis x globulus*. The peak numbers refers only to the chromatograms on the Figure 20.

Peak	Compound	Retention time (min)	*E.nitens* (mg compound /kg dry bark)	*E.grandis x globulus* (mg compound /kg dry bark)
1	Butan-1,3-diol	5.0	-	38.6
2	Lactic acid	5.1	12.4	3.7
3	Hexanoic acid	5.3	-	4.5
4	Diethylene glycol	11.1	2.4	1.8
5	Glycerol	12.8	4.4	15.4
6	Nonanoic acid	14.8	3.3	4.9
7	Decanoic acid	17.8	1.6	6.6
8	Vanillin	19.2	4.0	2.8
9	Docosan-1-ol	21.6	-	1.0
10	Dodecanoic acid	23.5	6.6	2.1
11	n.i. Sesquiterpenic alcohol	24.0	9.9	-
12	Vanillic acid	26.2	6.5	0.5
13	Tetradecanoic acid	28.6	7.3	1.4
14	Syringic acid	29.6	3.0	0.7
15	Pentadecanoic acid	31.0	4.5	1.0
16	Hexadecan-1-ol	31.6	5.4	32.2
17	Hexadecanoic acid	33.3	110.4	53.2
18	*(Z)*-9-Octadecen-1-ol	35.3	-	51.1
19	Heptadecanoic acid	35.5	5.6	10.4
20	Octadecan-1-ol	36.0	6.7	24.9
21	Linoleic acid	36.7	29.7	7.4
22	Oleic acid	36.9	26.2	1.2
23	Elaidic acid	37.1	2.8	
24	Octadecanoic acid	37.6	21.7	9.5
25	Eicosanoic acid	41.6	9.1	2.4
26	Heneicosanoic acid	43.5	4.8	-
27	Docosan-1-ol	43.9	2.6	-
28	Docosanoic acid	45.3	11.5	4.1
29	Tricosanoic acid	47.0	5.3	2.3
30	Tetracosan-1-ol	47.4	2.0	
31	Tetracosanoic acid	48.8	14.7	4.7
32	Stilbene [1*]	49.1	42.5	-
33	Pentacosanoic acid	50.4	7.5	2.2
34	Hexacosan-1-ol	50.7	5.1	1.3
35	22-Hydroxydocosanoic acid	51.5	-	1.2
36	Hexacosanoic acid	52.1	9.1	3.8
37	Octacosan-1-ol	54.3	14.5	7.9
38	Octacosanoic acid	55.9	8.2	3.1
39	*β* - Amyrin	57.4	330.8	40.9
40	*β* - Sitosterol	57.6	555.9	82.4
41	*β* - Sitostanol	57.7	52.8	7.9
42	Triacontan-1-ol	58.6	-	11.0
43	Triacontanoic acid	60.3	-	3.5
44	Oleanolic acid	62.2	86.3	8.0
45	Betulinic acid	62.6	91.2	7.5
46	Ursolic acid	63.1	68.4	13.0
47	3-Acetyloleanolic acid	63.6	23.0	4.4
48	3-Acetylursolic acid	64.8	18.6	9.7
	Total identified		**1733.1**	**524.1**
	Total non identified		**741.9**	**195.3**
	Total		**2475.1**	**719.5**

n.i. – non identified

1* - 4-hydroxy-3-[2-(4-hydroxy-3,5-dimethoxyphenyl)ethenyl]-5-methoxybenzaldehyde

The main chromatographic peaks were identified by comparing their mass spectra with the equipment mass spectral library (NIST mass spectral library 2005) and their characteristic retention times and fragmentation profiles with published data [19, 26, 52, 83, 91-95].

β-Sitosterol is the main sterol identified in the inner bark of some *Eucalyptus spp.* The mass spectra of TMS ethers of sterols exhibit easily discernible molecular ion peaks, or indirect information about their molecular weight can be obtained from the fragment ions formed by the loss of one methyl group $[M\text{-}CH_3]^+$, which is generally present in their spectra. The trimethylsilyl group strongly directs the fragmentation of the molecules and consequently, the mass spectra of sterols are usually characterized by the presence of abundant TMS-containing groups [83, 94, 95]. The ions at *m/z* 73 $[(CH_3)^3Si]^+$ and 75 $[[(CH_3)_2Si\text{-}OH]^+$ are present in the mass spectra of all TMS derivatives of sterols and in general provide negligible or no structural information. However, the TMS containing ions are in most cases of structural significance. For example, the ions at *m/z* 129 and $[M\text{-}129]^+$ which correspond to the loss of the TMS group, together with a three carbon fragment of ring A containing the C-1, C-2 and C-3, are characteristic of 3-hydroxy-Δ^5-sterols [26, 83, 93]. The ion corresponding to the 1,2-elimination of the trimethylsilanol group $[M\text{-}90]^+$ is another important structural ion [83]. Additionally, common fragmentations between the C_{13} and C_{17} and between the C_{14} and C_{15} bonds leading to the elimination of the D-ring (Figure 21) are also present. All the ions referred to above are clearly visible in the mass spectrum of the TMS derivative of *β*-sitosterol shown in Figure 21.

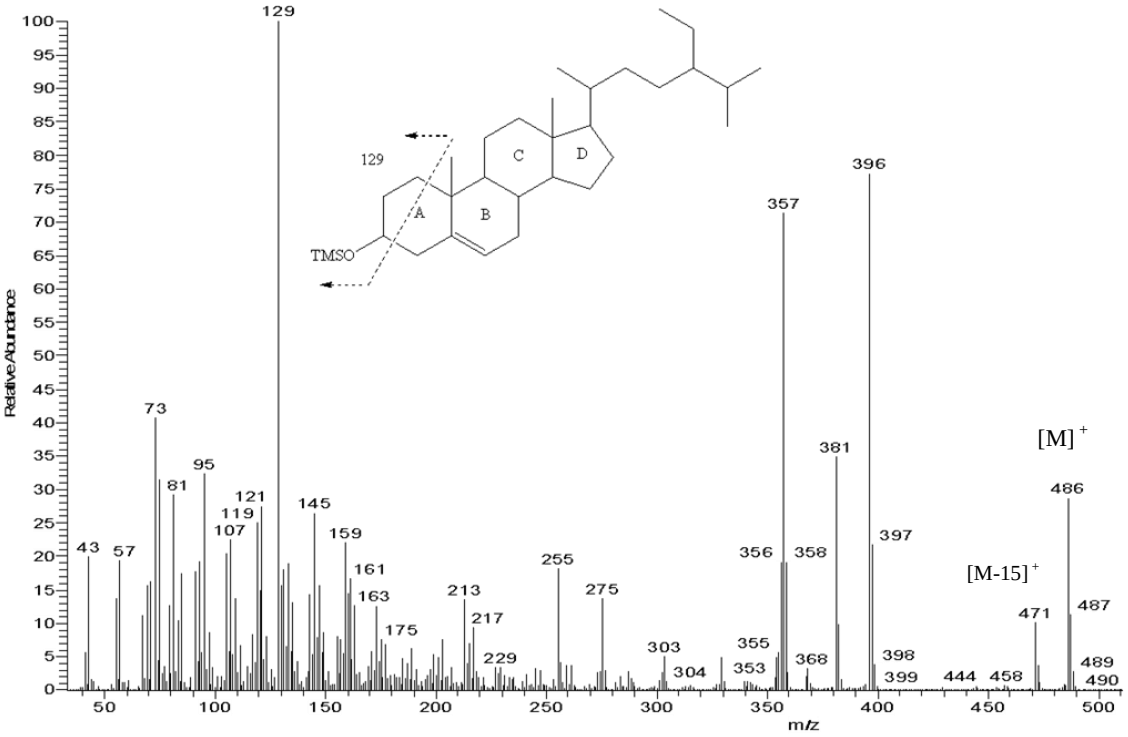


Figure 21 - Mass spectrum of the TMS derivative of *β*-sitosterol.

Fatty acids are also easily identified by GC-MS as TMS derivatives. Abundant ions arise from the derivatizing group itself at *m/z* 73 and 75. The ion $[M-15]^+$ and other abundant ions appear at *m/z* 117, 129, 132 and 145, being the last two due to the McLafferty type rearrangement [96, 97]. A mass spectrum of the TMS derivative of hexadecanoic acid is presented in (Figure 22) as an example.

Long chain aliphatic alcohols were identified by the high intensity of the *m/z* 75 and $[M-15]^+$ ions, that are characteristic of this type of compounds (Figure 23). Ions at *m/z* 89 and 103 $[(CH_3)_3SiOCH_2]^+$ are also usually present in the mass spectra of TMS derivatives of fatty alcohols (Figure 23) and help to distinguish them from the TMS derivatives of fatty acids [96, 97].

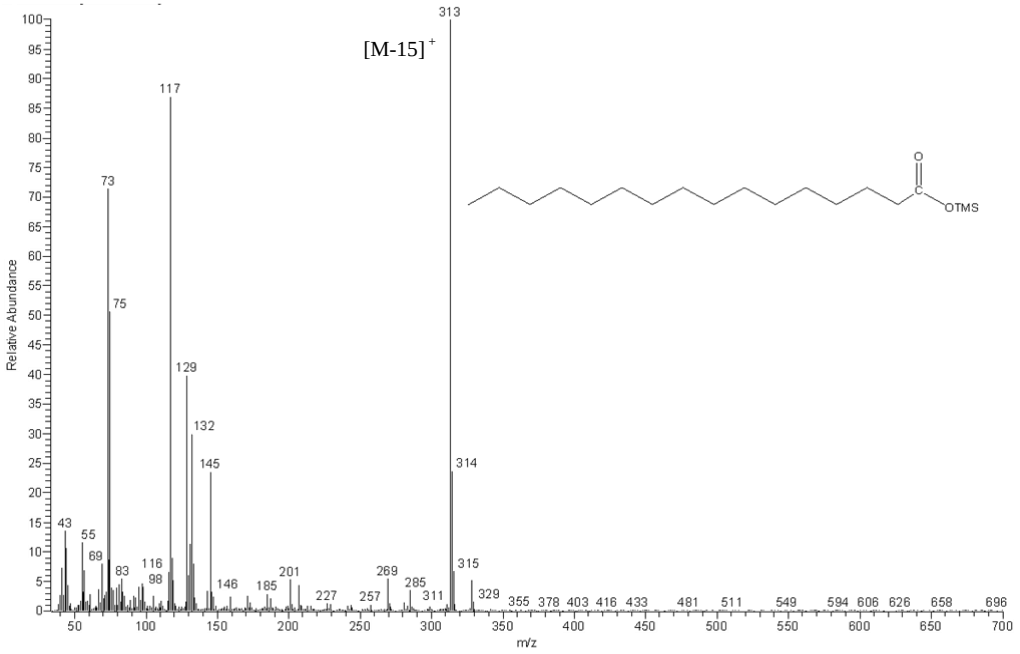


Figure 22 - Mass spectrum of the TMS derivative of hexadecanoic acid.

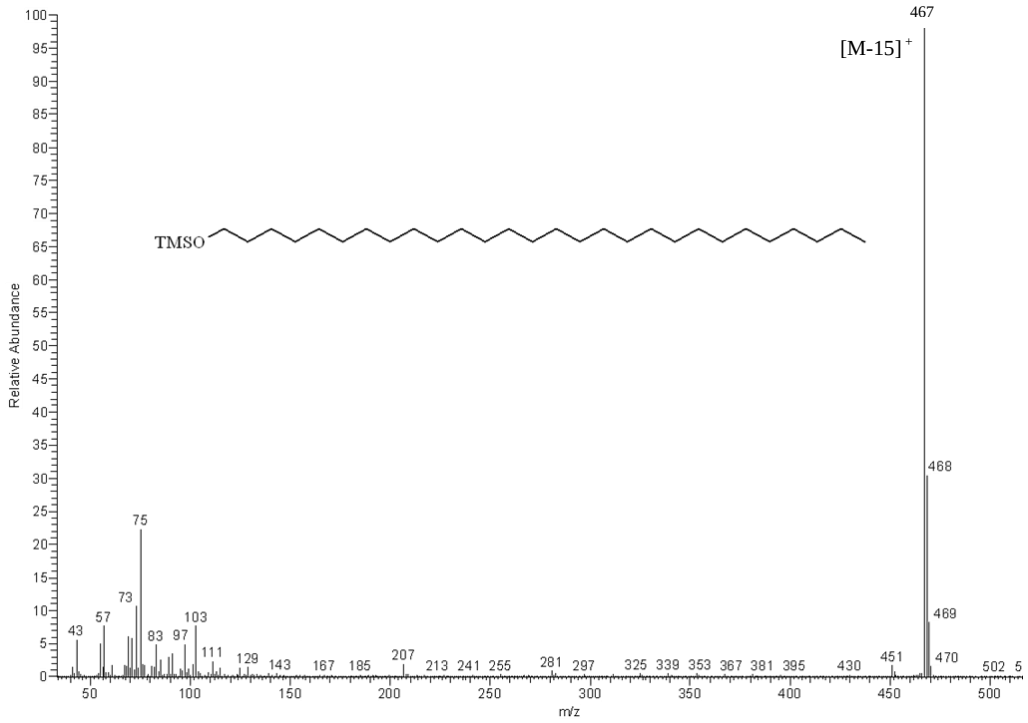


Figure 23 - Mass spectra of the TMS derivative of octacosan-1-ol.

4.1.3. CHARACTERIZATION OF OUTER BARK LIPOPHILIC EXTRACTIVES

Typical GC-MS chromatograms obtained of the derivatized dichloromethane extracts of *E. nitens* and *E. grandis x globulus* outer barks are presented in Figure 24 and the lipophilic components (mg of compound/kg of dry bark) identified in these fractions are shown in Table 3.

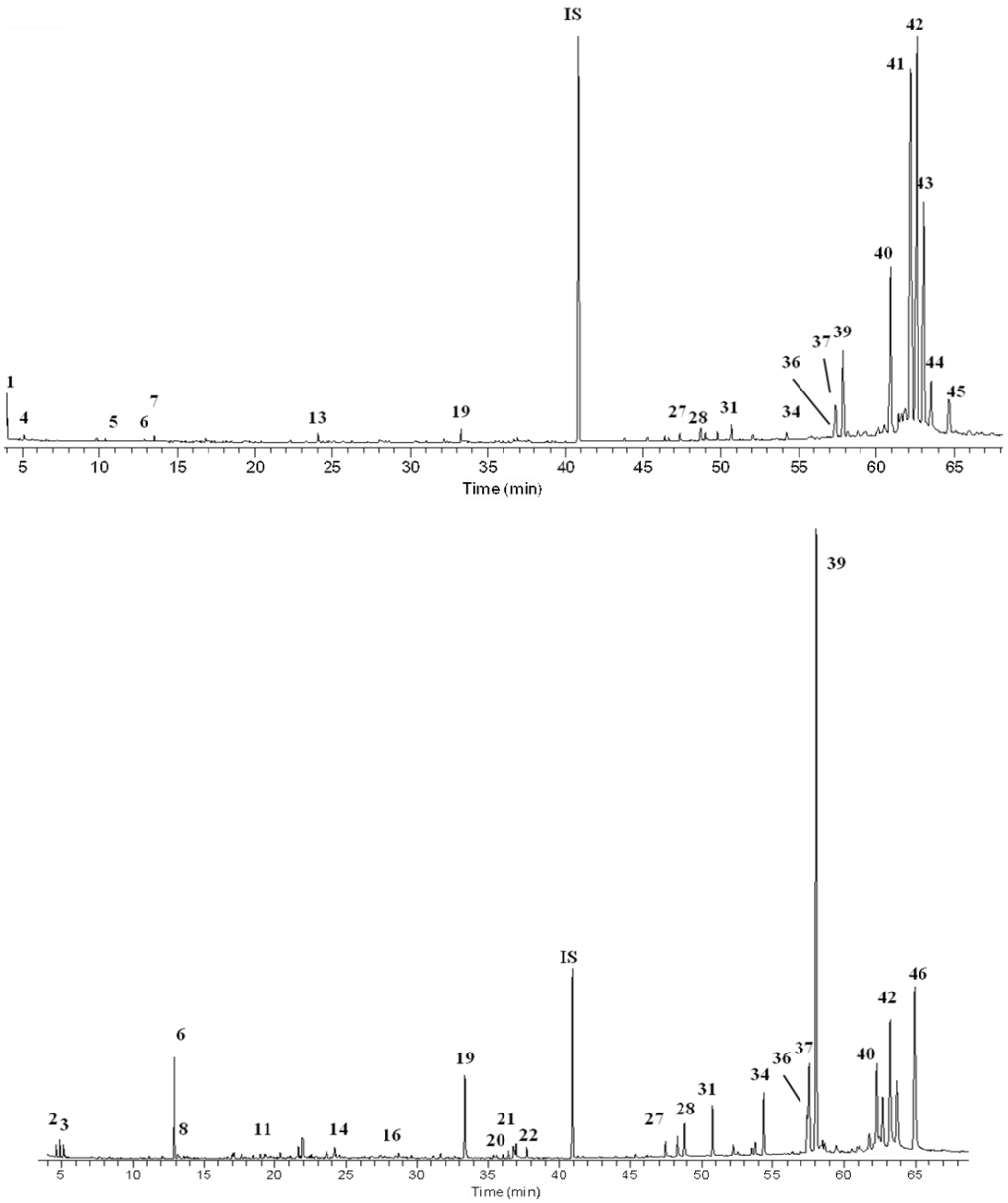


Figure 24 - Typical GC-MS chromatograms of the derivatized dichloromethane extracts of *E. nitens* (upper) and *E. grandis x globulus* (lower) outer barks.

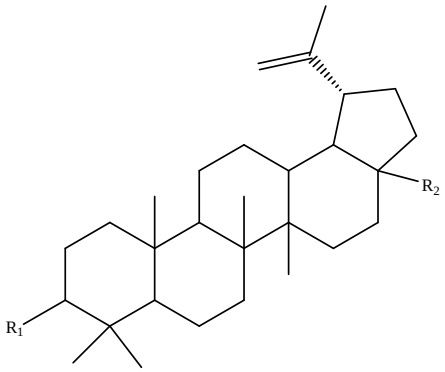
Table 3 - Lipophilic components (mg of compound/kg of dry bark) identified in the dichloromethane extract of the outer bark of *E. nitens* and *E. grandis x globulus*. The peak numbers refers only to the chromatogram on the Figure 24.

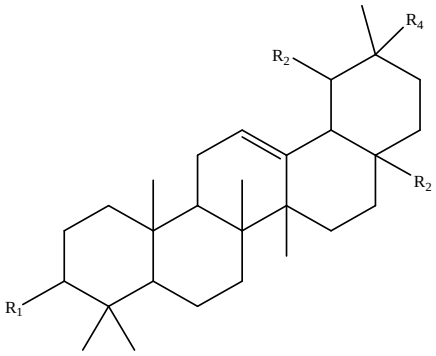
	Compound	Retention time (min)	*E.nitens* (mg compound /kg dry bark)	*E.grandis x globulus* (mg compound /kg dry bark)
1	Eucalyptol	4.0	148.8	-
2	Propane-1,2-diol	4.8	-	37.6
3	Butane-2,3-diol	5.0	-	65.9
4	Lactic acid	5.1	48.4	8.76
5	n.id. Monoterpene	10.4	22.9	-
6	Glycerol	12.8	17.8	29.2
7	α-Terpeniol	13.5	44.5	-
8	Butane-1,2,3-triol	13.7	-	14.0
9	Aromadendrene	16.8	34.4	-
10	Allo-aromadendrene	17.4	4.2	-
11	Vanillin	19.6	-	28.6
12	n.i. sesquiterpenic alcohol	22.2	10.9	-
13	n.i. sesquiterpenic alcohol	24.1	56.4	
14	Syringaldehyde	24.4	-	58.7
15	Nonadienoic acid	27.5	-	14.2
16	Tetradecanoic acid	28.2	-	17.3
17	Pentadecanoic acid	31.2	-	8.7
18	Hexadecan-1-ol	31.8	-	33.6
19	Hexadecanoic acid	33.3	110.2	89.3
20	Linoleic acid	36.7	28.6	31.0
21	Oleic acid	36.9	37.1	19.5
22	Octadecanoic acid	37.6	12.3	17.6
23	Docosan-1-ol	43.8	38.6	-
24	Docosanoic acid	45.3	36.2	12.1
25	n.i. alcane	46.4	42.5	-
26	n.i. stilbene	46.6	25.6	-
27	Tetracosan-1-ol	47.3	65.6	30.6
28	Tetracosanoic acid	48.7	103.5	136.8
29	Stilbene [1*]	49.0	75.8	-
30	n.i. alcane	49.8	93.0	-
31	Hexacosan-1-ol	50.7	134.4	138.5
32	Hexacosanoic acid	52.1	55.3	93.5
33	α-tocopherol	53.9	-	15.5
34	Octacosan-1-ol	54.2	72.2	224.6
35	Octacosanoic acid	55.9	36.9	18.8
36	β - Amyrin	57.3	90.9	103.2
37	β - Sitosterol	57.4	407.4	267.0
38	Lupeol	58.2	105.9	
39	**Methyl-3-hydroxyolean-18-en-28-oate**	58.4	1040.9	**3216.8**
40	Betulonic acid	60.9	2436.5	71.1
41	Oleanolic acid	62.2	**7250.1**	916.3
42	Betulinic acid	62.6	6621.0	626.0
43	Ursolic acid	63.1	3537.1	1279.0
44	3-Acetyloleanolic acid	63.5	1101.2	715.1
45	3-Acetylbetulinic acid	64.4	-	28.2
46	3-Acetylursolic acid	64.7	640.6	1598.1
	Total identified		**24336.1**	**10000.6**
	Total non identified		**2076.6**	**989.4**
	Total		**26412.7**	**10990.0**

n.i. – non identified

1* - 4-hydroxy-3-[2-(4-hydroxy-3,5-dimethoxyphenyl)ethenyl]-5-methoxybenzaldehyde

Triterpenic acids with lupane, ursane and oleanane skeletons, namely betulonic **(40)**, ursolic **(43)**, betulinic **(42)** oleanolic **(41)**, 3-acetylursolic **(46)** and 3-acetyloleanolic **(44)** acids (Figure 25), are clearly the main lipophilic extractives found in *E. nitens* outer bark, representing more than 21.6 g/kg of outer bark, (Table 3). This outer bark extract composition is very similar to those of other *Eucalyptus spp.* already reported in the literature [14, 26, 89].





R_1=OH, R_2=CH_3 Lupeol

R_1=OH, R_2=COOH Betulinic acid

R_1=OC(O)CH_3, R_2=COOH 3-acetyl betulinic acid

R_1= =O, R_2=COOH Betulonic acid

R_1=OH, R_2=CH_3, R_3=H, R_4= CH_3 β-amyrin

R_1=OH, R_2=CH_3, R_3=CH_3, R_4= H α-amyrin

R_1=OH, R_2= COOH, R_3= H, R_4= CH_3 Oleanolic acid

R_1= OC(O)CH_3, R_2=COOH, R_3= H , R_4= CH_3 3-acetyloleanolic acid

R_1=OH, R_2=COOH, R_3= CH_3 , R_4= H Ursolic acid

R_1= OC(O)CH_3, R_2= COOH, R_3= CH_3 , R_4= H 3-acetylursolic acid

Figure 25 - Structures of triterpenoids identified in *Eucalyptus nitens* outer bark.

The TMS derivatives of triterpenic acids displayed also typical mass spectra. Oleanolic and ursolic acids show the most important signals at *m/z* 600 $[M]^+$, 585 $[M-CH_3]^+$, 510 $[M-TMSOH]^+$, 495 $[M-TMSOH-CH_3]^+$, 482 $[M-TMSOOCH]^+$, 393 $[M-TMSOH-TMSOOC]^+$ and 392 $[M-TMSOH-TMSOOCH]^+$ (Figure 26). Triterpenes that contain a C_{12}-C_{13} double bond undergo a retro-Diels-Alder reaction to form fragments containing the A, B and part of C-rings and the other part of C, E and D-rings (Figure 26) originate fragments at *m/z* 320, 307, 279, 203 and 189, which are characteristic ions [91, 92, 98, 99].

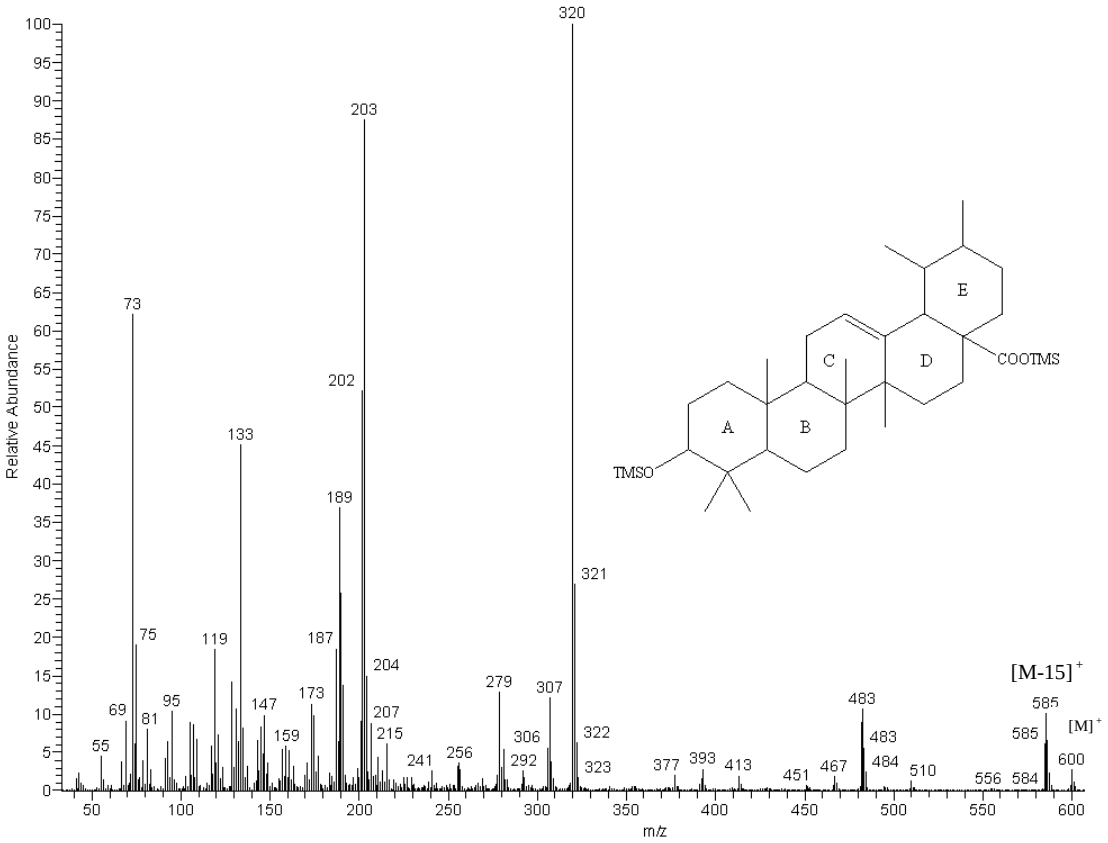


Figure 26 - Mass spectrum of the TMS derivative of ursolic acid.

Betulinic and betulonic acids were also found in considerable amounts in the extract. Betulinic acid present a fragmentation pattern similar to those of ursolic and oleanolic acids [92], with a molecular ion at *m/z* 600. However, an intense peak at *m/z* 189 is characteristic of this lupane type structures. Peaks at 585 $[M\text{-}CH_3]^+$, 510 $[M\text{-}TMSOH]^+$, 482 $[M\text{-}TMSOOCH]^+$, 393 $[M\text{-}TMSOH\text{-}TMSOOC]^+$ and *m/z* 73 $[TMS]^+$ [83, 92] are also present in the mass spectra of these compounds (Figure 27). The mass spectra of the TMS derivative of betulonic acid differ from the betulinic acid derivative in the molecular ion, with a peak at *m/z* 526 due to the carbonyl group instead of the hydroxyl group in the C_3 position [92].

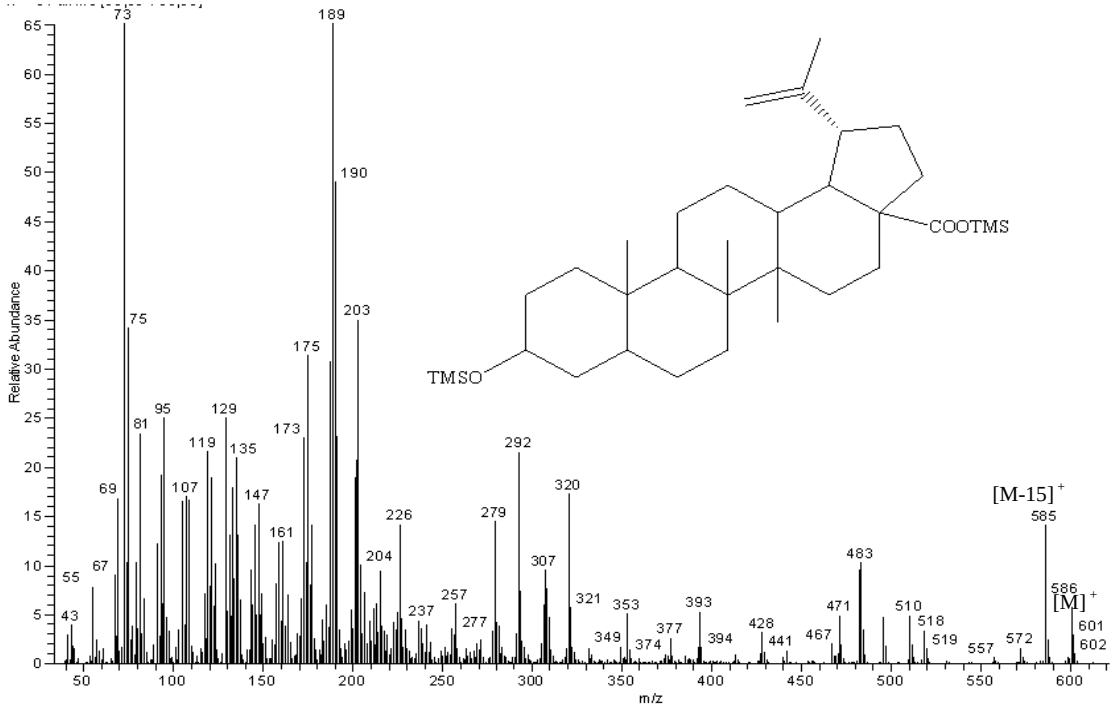


Figure 27 - Mass spectra of the TMS derivative of betulinic acid.

The acetylated triterpenoids of oleanolic and ursolic acids were also identified. The main difference between their spectra is the molecular ion, that in the TMS acetylated derivatives occur at *m/z* 570 (Figure 28) instead of *m/z* 600. This difference is due to the presence of the acetyl group in the C_3 position.

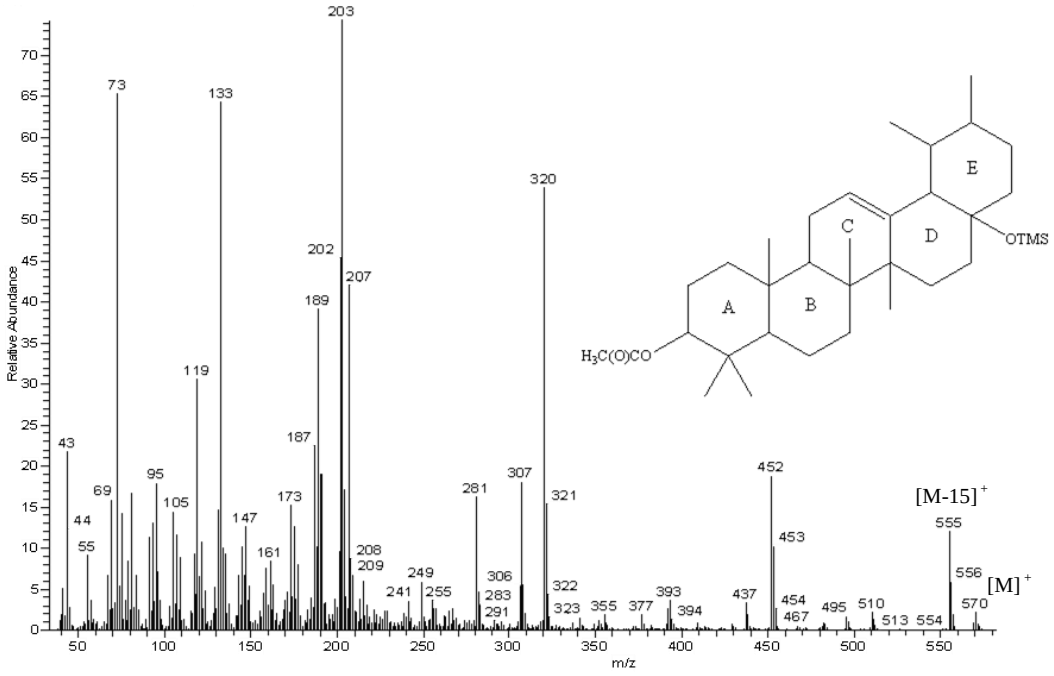
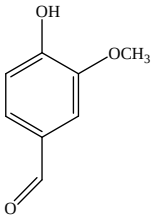


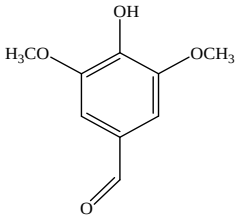
Figure 28 - Mass spectrum of the TMS derivative of 3-acetylursolic acid.

Monoterpenes and sequiterpenes were also identified in *E. nitens* outer bark, with eucalyptol **(1)** (148.8 mg/kg), *α*-terpeniol **(7)** (44.5 mg/kg) and aromadendrene **(9)** (34.4 mg/kg) as some examples of these two families. Smaller amounts of long chain aliphatic alcohols (0.3 g/kg), fatty acids (0.4 g/kg) and sterols (0.5 g/kg) were also identified in this extract.

The *E. grandis x globulus* outer bark shows a profile somewhat different from what is common for the outer bark fractions of *Eucalyptus* species. The main difference lies in the fact the most abundant compound is not a typical triterpenic acid, such as oleanolic, ursolic or betulinic acids, as it happens in the other species. The most abundant compound of this outer bark fraction was identified as **methyl-3-hydroxyolean-18-en-28-oate (39) (3 g/kg)**, an oleanane structure type triterpene that was never identified in *eucalyptus* bark. This compound was isolated and his characterization is described below (point 4.2). However, the remaining composition of this extract is similar to other species, with triterpenic acids, oleanolic **(41)**, ursolic **(43)** and betulinic **(42)** acids being also detected. In this fraction some aromatic compounds (Figure 29) such as vanillin **(11)** (28.6 mg/kg), syringaldehyde **(14)** (58.7 mg/kg) and *α*-tocopherol **(33)** (15.5 mg/kg) and several fatty acids and long chain aliphatic alcohols were also identified. The aromatic compounds were identified as TMS derivatives, based on the intense molecular ion and $[M-15]^+$ peaks. Abundant peaks corresponding to the $[M-30]^+$ fragmentation (loss of an additional methyl group) are also typical of this family of compounds [100].



Vanillin



Syringaldehyde

Figure 29 - Structures of two phenolic compounds identified in the outer bark fraction of *E. grandis x globulus*

Among the six *Eucalyptus* barks species already studied in our group [26, 89, 90], *E. nitens* outer bark reveals to be the one with the highest triterpenic acids content, with 21.6 g/kg of bark. As shown in Table 4, only *E. globulus* has similar contents of triterpenic acids with 21.3 g/kg.

Therefore, regarding these values, *E. nitens* and *E. globulus* outer barks seem to be the most promising raw materials for the exploitation of bark residues as important sources of bioactive triterpenic acids, in an integrated biorefinery perspective within the pulp and paper industry.

Table 4 - Major triterpenic compounds identified in *Eucalyptus* species outer barks (g/kg of dry bark).

Compound[a]	*Eucalyptus spp.*					
	E. globulus	*E. nitens*	*E. maidenii*	*E. urograndis*	*E. grandis*	*E grandis x globulus*
Betulonic acid	2.6	2.4	1.0	-	-	-
Oleanolic acid	4.1	8.4	1.7	1.2	0.7	1.6
Betulinic acid	2.6	6.6	2.0	1.4	2.1	0.6
Ursolic acid	12.1	4.2	3.6	1.9	2.4	2.7
Total	21.3	21.6	8.4	4.5	5.1	4.9

[a]- including 3-acetyl derivative.

In the Table 5 are represented the main families of compounds found in the bark of the two species studied compared with *E. globulus*, since it is the most used species in the Iberian Peninsula.

Table 5 - Major families of compounds identified in *Eucalyptus* species (mg/kg of dry bark)

	Inner			**Outer**		
mg/kg	*E. nitens*	*E. grandis x globulus*	*E. globulus*	*E.nitens*	*E. grandis x globulus*	*E. globulus*
FA	305	136	319	468	483	1232
LCAA	38	208	289	311	594	1328
ST	602	90	571	407	267	1114
TT	767	110	720	21783	8554	21346

4.2. CHARACTERIZATION OF METHYL-3-HYDROXYOLEAN-18-EN-28-OATE

The identification of **methyl-3-hydroxyolean-18-en-28-oate** (methyl morolate) was possible based on the analysis of it mass spectra, ^{1}H and ^{13}C NMR spectra and confirmed by DEPT (Distortionless Enhancement by Polarization Transfer) (135 and 90), 2D NMR: COSY ($^{1}H/^{1}H$), HSQC (^{1}J - $^{1}H/^{13}C$), HMBC ($^{2/3}J$- $^{1}H/^{13}C$), and Nuclear Overhauser Effect Spectroscopy (NOESY). The key NMR features of this compound, as well as the connectivities found in 2D spectra, are shown and discussed in the next paragraphs and figures.

The analysis of the **methyl-3-hydroxyolean-18-en-28-oate** TMS derivative mass spectrum (Figure 30), showed a molecular ion at *m/z* 542, corresponding to a molecular weight of 470 g/mol of the non-derivatized compound, as confirmed by ESI-MS analysis. The mass spectrum is similar to those found for other triterpenic acids. The main difference is the molecular ion peak that, in triterpenic acids, like ursolic or oleanolic acid, is found at *m/z* 600. This difference suggests the presence of a methyl ester instead of a TMS ester. This was confirmed by the alkaline hydrolysis that results in a compound with a molecular ion at *m/z* 600 (TMS derivative).

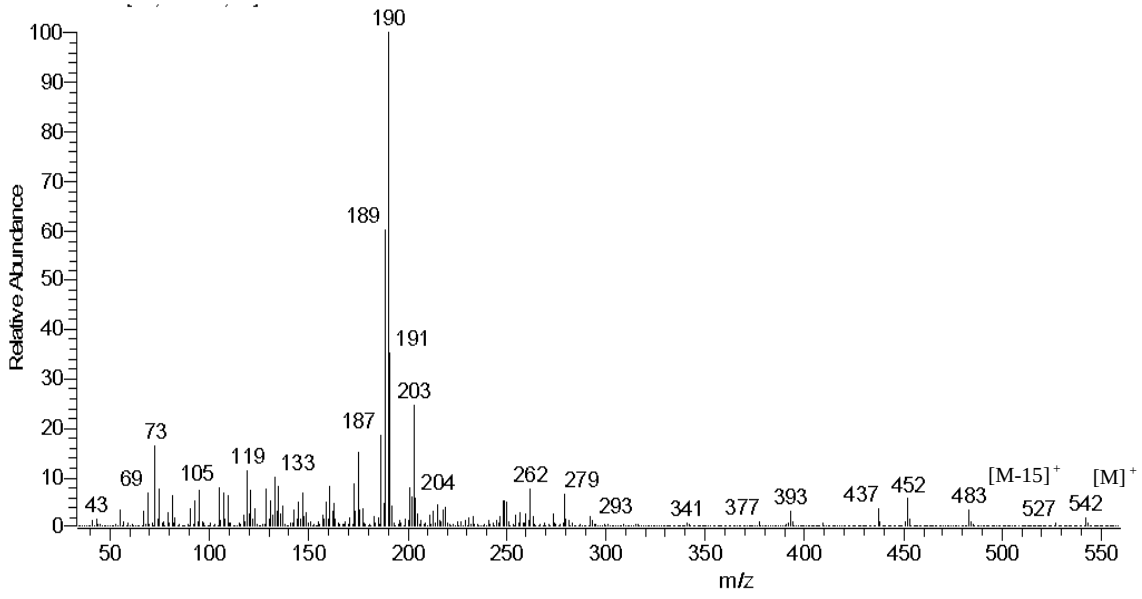


Figure 30 - Mass spectrum of methyl-3-hydroxyolean-18-en-28-oate TMS derivative.

Despite the identification of the peaks as a methylated compound, its retention time and the mass spectra together with that of the hydrolysed compound showed that this was a compound not reported in our previous works. Additionally, the NMR profile of the isolated compound was not coincident with compounds previously identified in our group. However, in some earlier studies, **morolic acid** (3-*β*-hydroxyolean-18-en-oic acid) (Figure 31) have been already isolated from the bark of *Eucalyptus papuana* and *Eucalyptus grossa* [101, 102]. This triterpenic acid differs from oleanolic acid in the location of the double bond, which in this case is located in the C_{18}, whereas in oleanolic acid is in C_{12}. This variation could lead to the different pattern observed in the mass spectra and in some differences in the NMR data found.

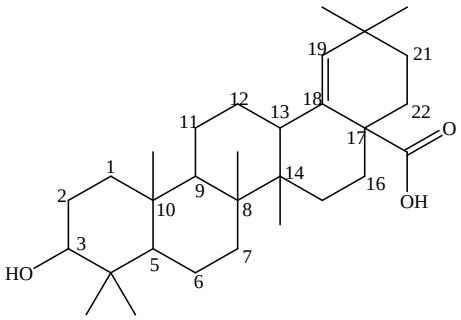


Figure 31 – Structure of morolic acid found in the bark of *E. grossa.*

Budzikiewicz *et al.* [103] have elucidated the characteristic mass fragment patterns of the methyl morolate (Figure 32). Cleavage of the ring C yields the characteristic fragment *a*, with a TMS group, at *m/z* 279 visible in the mass spectra. As described for 12-oleananes, the right-hand portion of the molecule (fragment *b*, *m/z* 262) is formed by the cleavage of 11-12 bond. Fragment *b* suffers further loss of the C_{17} substituent giving a fragment *c* with *m/z* 203. In 18-oleananes, *m/z* 189 is the most abundant fragment, followed by 203, but in this case, is the *m/z* 190 the most abundant fragment. The same author, reported that an alternate fission of the 11-12 bond yields two species, *d* and *d'* with *m/z* 248 and 249 respectively. After that, by losing of C_{17} substituent, two fragments are originated, *e* and *e'*, with *m/z* 189 and 190, respectively [99].

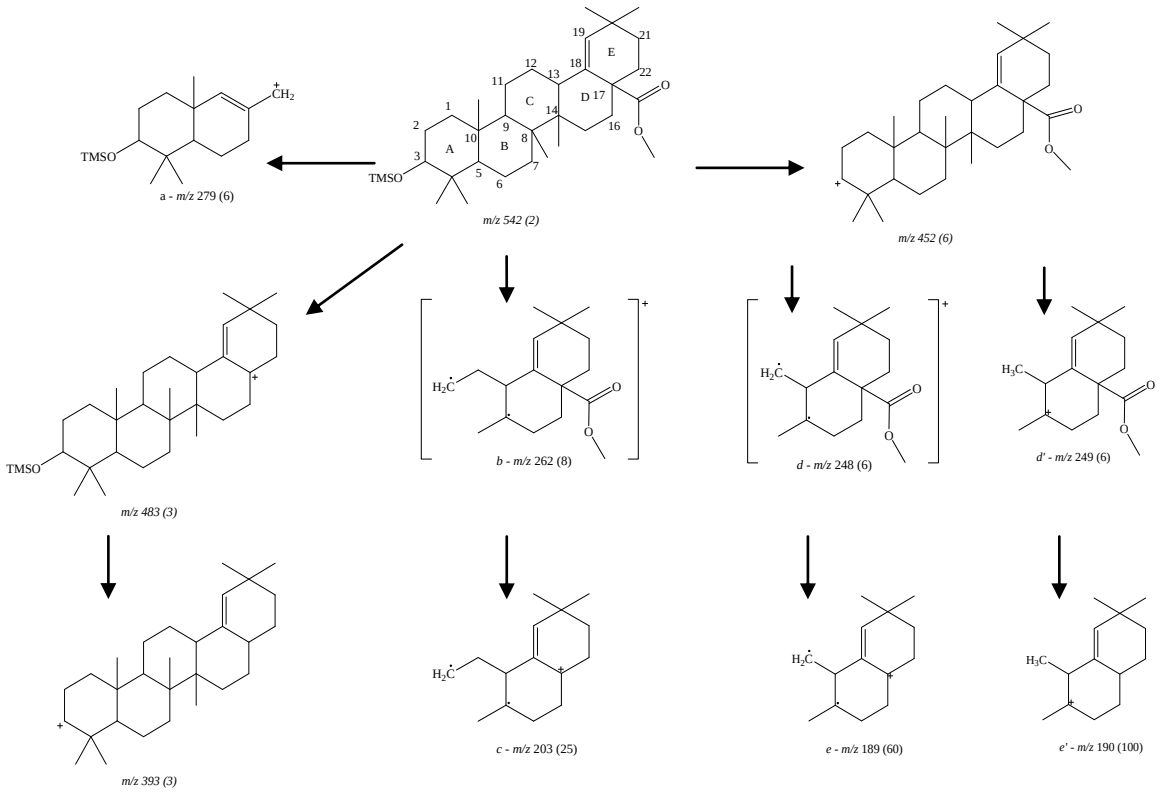


Figure 32 - Main cleavages and relative abundances of TMS derivative of methyl morolate under EI conditions.

4.2.1. ^{1}H NMR

As expected for triterpenic structures, the ^{1}H NMR spectrum (Figure 33) revealed a large number of signals in the aliphatic region, between δ 0.2 – 1.8 ppm, were methyl groups are well visible. The triplet found at δ 3.38 ppm, is assigned to C<u>H</u>-OH. In the HSQC spectrum, this proton correlates with a carbon at δ 76.2 ppm, that is the C_3 of the structure. The singlet at δ 3.69 ppm is assign to a COOC<u>H</u>$_3$ proton, confirming the presence of a methyl ester group. This resonance correlates with a carbon at δ 51.9 ppm in HSQC spectrum. The doublet at δ 5.12 ppm (J=1.5Hz) corresponds to a double bond proton. Low intensity resonances at 1.26 (t), 2.05 (s) and 4.12 (q) are due to traces of ethyl acetate used in the purification.

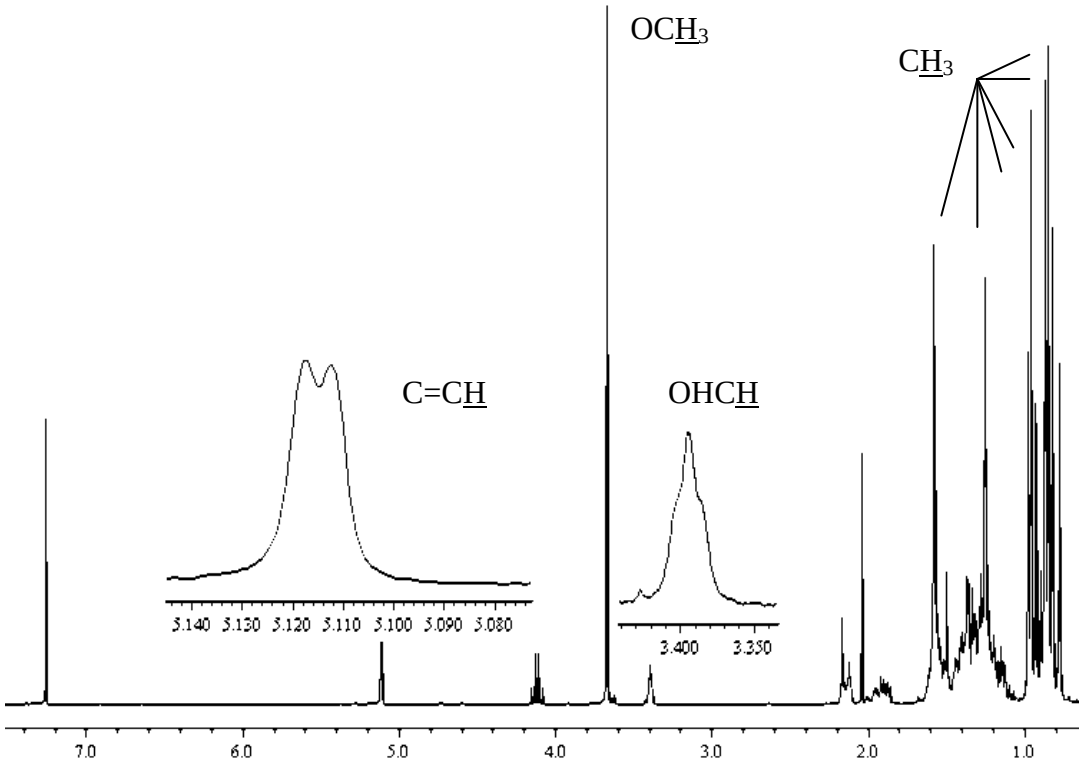


Figure 33 - ^{1}H NMR spectrum of methyl-3-hydroxyolean-18-en-28-oate.

4.2.2. ^{13}C NMR

In the ^{13}C spectrum (Figure 34), most of the resonances are in the aliphatic region (δ 15 and 50 ppm). As expected, two resonances appear at δ 132.5 and 137.2 ppm confirming the existence of a double bond. Based on DEPT analysis, those resonances are tertiary and quaternary, respectively. Furthermore, the tertiary nature of the carbon assigned to the resonance at δ 132.5 ppm was confirmed based on the HSQC experiments, through the correlation with the proton resonance at δ 5.12 ppm. The resonance at δ 76.3 ppm is assigned to C_3 also confirmed by the HSQC experiments. Finally, the resonance at δ 177.3 ppm is assigned to the COOMe carbon.

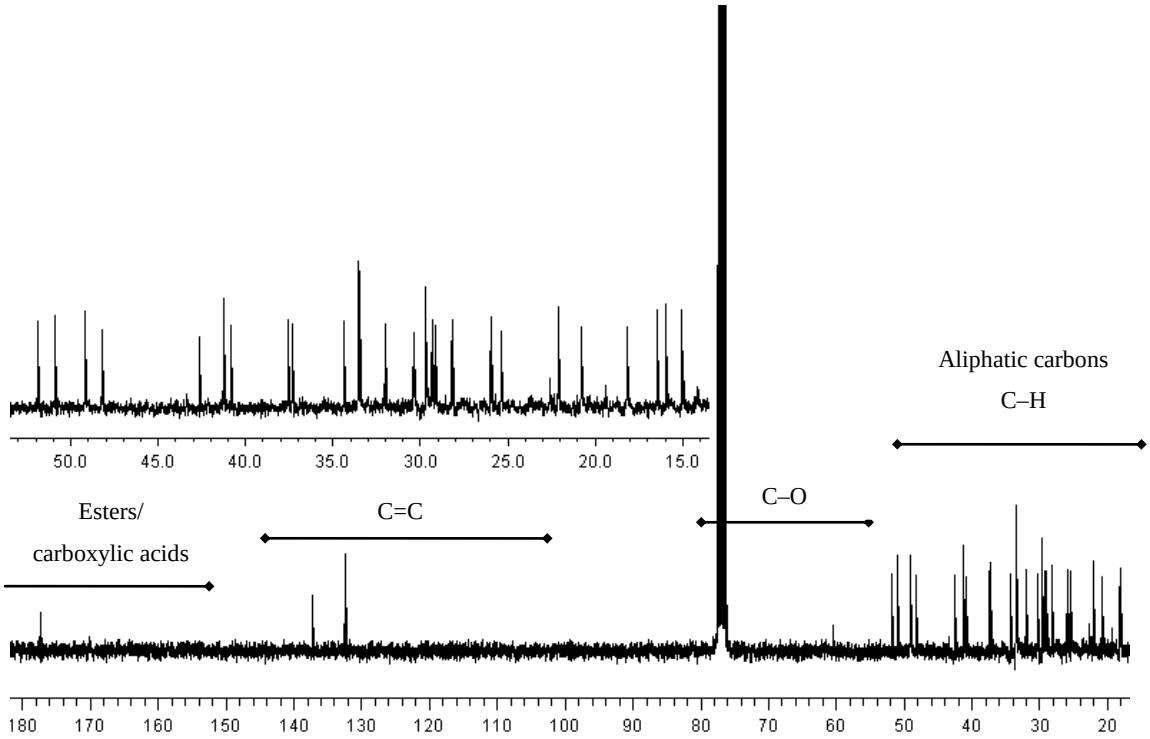


Figure 34 - ^{13}C NMR spectrum of methyl-3-hydroxyolean-18-en-28-oate.

Although most the MS and NMR data are in agreement with the literature in several points, the results obtained in this study were not conclusive in what concerns to the position of the double bond in the structure. Also, in the ^{1}H NMR spectra where the vinilic proton appears as a doublet (Table 6), in the literature it is always reported as singlet [104-106]. Therefore, in addition to ^{1}H NMR and ^{13}C NMR, several 2D experiments were required to confirm the structure.

Table 6 - ^{1}H and ^{13}C NMR chemical shifts of methyl-3-hydroxyolean-18-en-28-oate in $CDCL_3$ with TMS as internal standard.

	ppm (δ)	
#	^{1}H	^{13}C
3	3.38	76.2
18	-	137.2
19	5.13	132.5
31	3.69	51.8

4.2.3. 2D NMR STUDIES

In the COSY spectrum, the resonance at δ 5.12 ppm correlates with a resonance at δ 2.2 ppm (Figure 35). This resonance at δ 2.2 ppm correlates in HSQC with a carbon at δ 41.2 ppm, that in DEPT experience show to be a tertiary carbon. This information confirms the allylic coupling between the proton at C_{19} and the proton existing in the tertiary carbon at C_{13} (Figure 36). This information has not been described in previous studies as mentioned before.

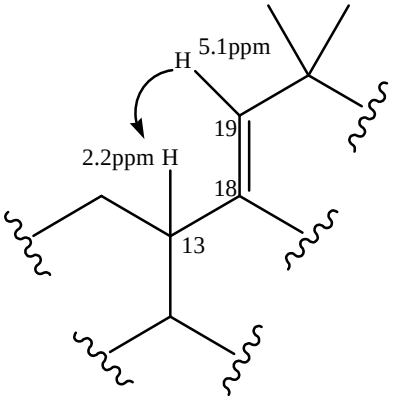


Figure 35 - Allylic correlation present in the COSY spectrum.

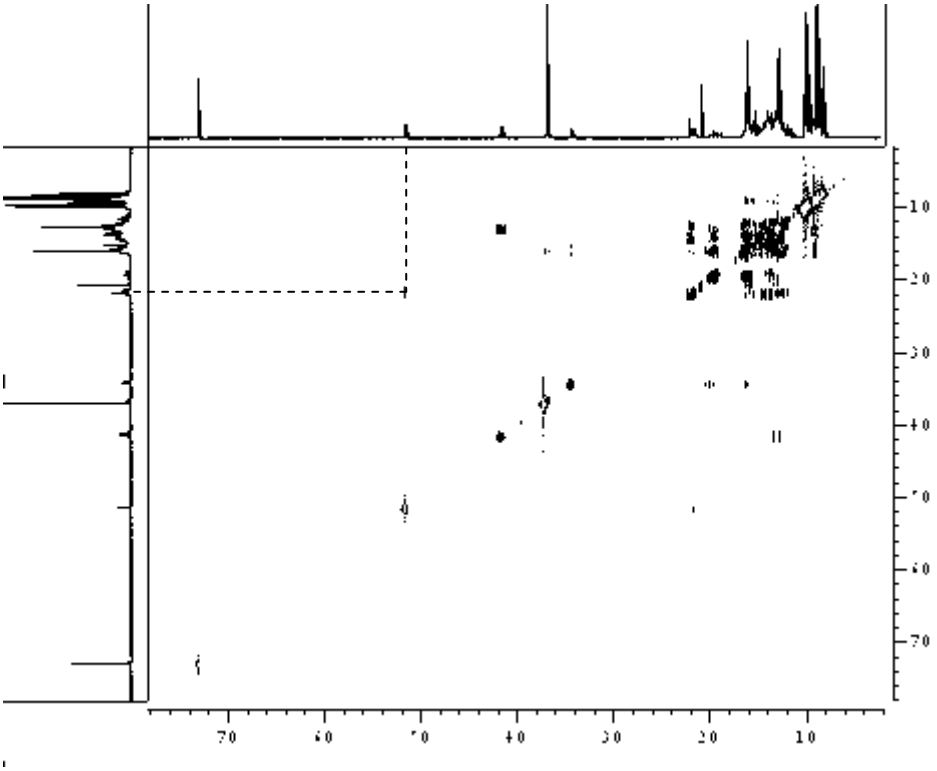
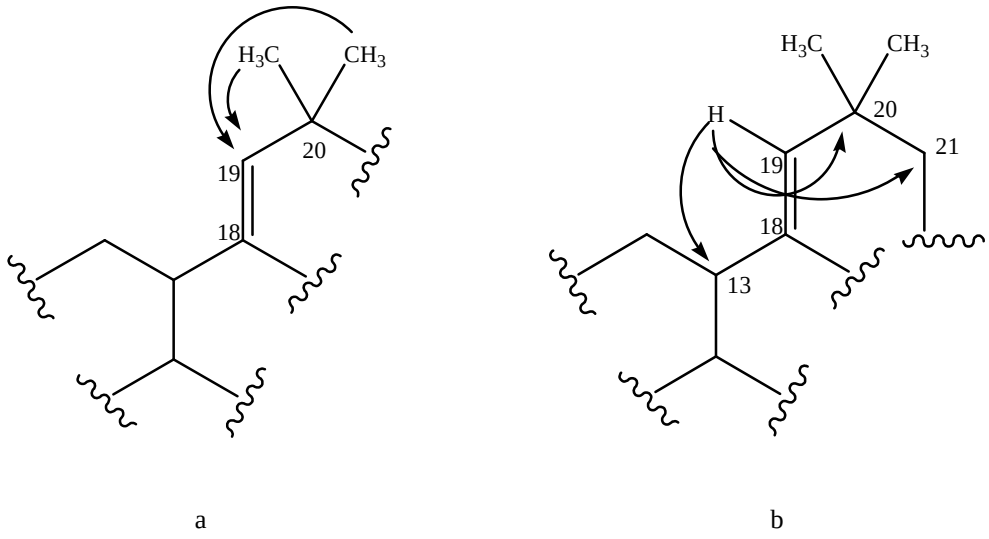


Figure 36 - COSY spectrum of methyl-3-hydroxyolean-18-en-28-oate.

The first HMBC studies were optimized for J^3 coupling at 7 Hz. The coupling constant is affected by substituents or double bonds, so 2-bond (J^2) correlations are sometimes observed.

In the HMBC spectrum, the protons of the methyl groups of the 20-C<u>H</u>$_3$ correlate with C_{19} (Figure 39), confirming that the tertiary carbon (DEPT) of the double bond is connected to a carbon that has two methyl groups attached (Figure 37a).

To confirm this scenario, in the same HMBC spectrum, H-19 should correlate with the two carbons from the methyl groups. However, this is not visible in this spectrum, maybe due to some interference in the coupling constant. So, 2 Hz experiments should be carried out to confirm this hypothesis. Other important correlations from this proton are those with a secondary carbon (C_{21}), with a tertiary carbon (C_{13}) and with a quaternary carbon (C_{20}) (Figure 37b). These attributions were confirmed with the DEPT and HSQC experiments.



a b

Figure 37 - HMBC correlations of methyl-3-hydroxyolean-18-en-28-oate.

In the HMBC spectrum, the triplet at δ 3.38 ppm assigned to H-3, which is linked to C3-OH, correlates with two carbons from methyl groups, confirming the usual position of the hydroxyl group and also the position of two methyl groups in C_4 position (Figure 38).

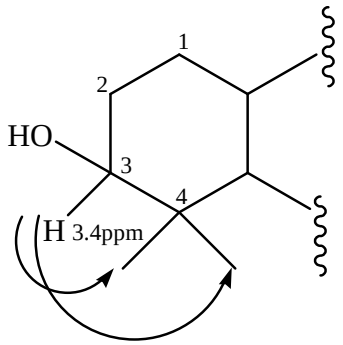


Figure 38 - HMBC correlations of methyl-3-hydroxyolean-18-en-28-oate.

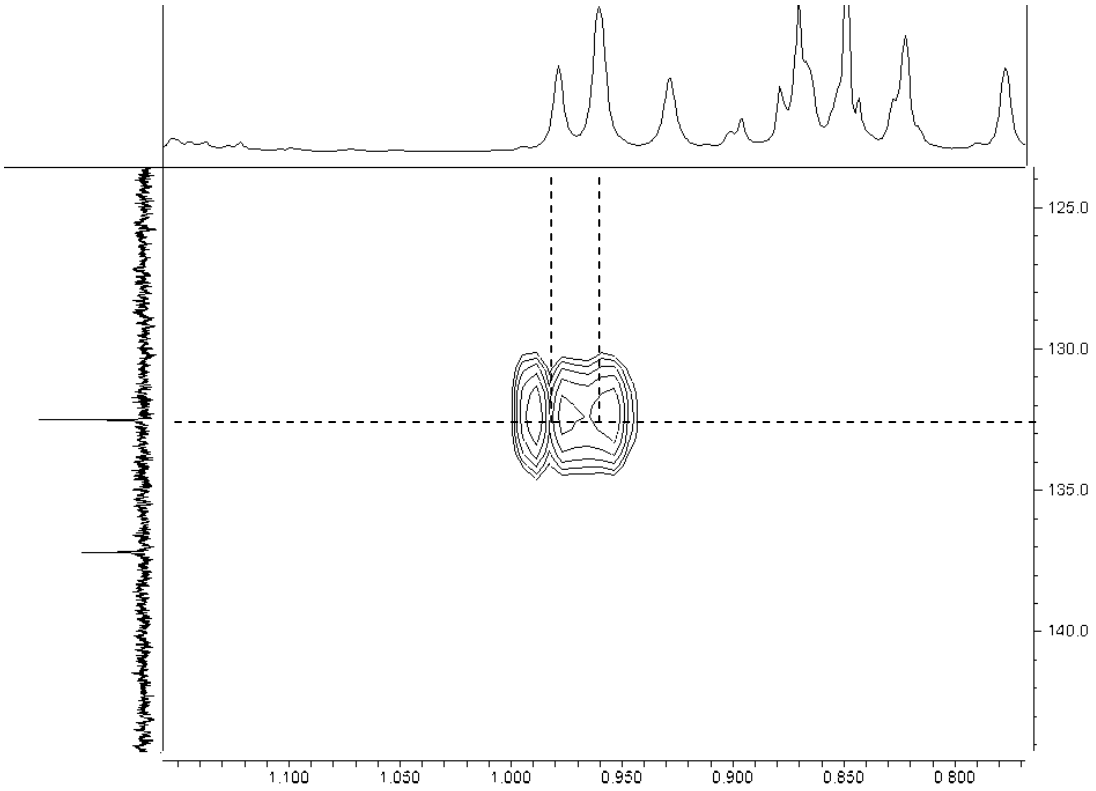


Figure 39 - HMBC correlation of methylic protons with carbon from double bond.

The NOESY experiment correlates all protons which are close in space. In this case, some visible correlations can confirm some correlations discussed earlier. For example, the correlation between the proton from C_3 with protons from two methyl groups (Figure 40).

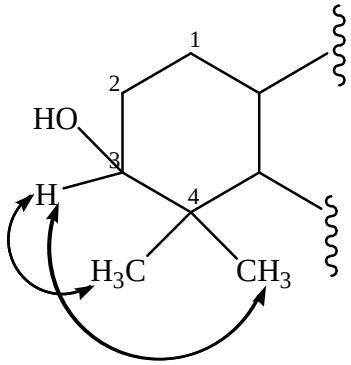


Figure 40 - NOESY correlation between proton from HCOH with methylic protons from $C_{4.}$

4.3. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY

Solid/liquid extractions of the total bark of *E. nitens* and *E. grandis x globulus* were performed. The extraction yields of the studied bark extracts and the corresponding total phenolic contents (TPC) are shown in Table 7. The extraction yields were obtained after removal of the dichloromethane soluble fraction, which accounted for 0.68% of bark weight for *E. nitens* and 0.69% for *E. grandis x globulus*.

Table 7 – Yield and total phenolic content of the two *Eucalyptus* species studied.

spp	Extraction yield (%)	TPC (mg GAE[a]/g of extract)	TPC (mg GAE[a]/g of bark)
E. nitens	9.01	542.9±6.8	48.9±0.6
E. grandis x globulus	11.0	316.5±2.5	34.9±0.3

[a]GAE – Gallic acid equivalents / Values are expressed as mean±standart deviation

The MeOH:H_2O extraction yield of 9.01 % for *E. nitens* is similar to the yield of 9.28% for *E. globulus* reported earlier [61]. *E. grandis x globulus* shows higher extraction yields, accounting for 11.03 %. The TPC of the extracts of *E. nitens* and *E. grandis x globulus* bark, determined by Folin-Ciocalteu method, accounted for 542.9±6.8 mg GAE g^{-1} for *E. nitens*, and 316.5±2.5 mg GAE g^{-1} for *E. grandis x globulus*. These values are in the same order of those found for *E. globulus* (413.8±5.3 mg GAE g^{-1} [61]).

Table 8 - Antioxidant activity of the two bark extracts studied

spp	Extraction yield (%)	IC_{50} (µg/mL)	mg AAE[a] /g of bark
E. nitens	9.01	5.63±0.05	33.89±0.32
E. grandis x globulus	11.0	5.13±0.18	44.59±0.42

[a]AAE – Acid ascorbic equivalents / Values are expressed as mean±standart deviation

Table 8 shows the antioxidant activity of the studied extracts, expressed in terms of the amount of extract required to reduce into 50% the DPPH concentration (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) on a dry bark basis (mg AAE/g dry bark). Taking into account for comparative purpose the IC_{50} value of ascorbic acid to be about 2.1 µg/mL, these extracts have revealed an antioxidant activity in the range of ascorbic acid. In general, *E. globulus* (19.6 mg AAE /g of bark[107]) shows lower antioxidant activity than *E .nitens* and *E. grandis x globulus*.

5. CONCLUSIONS

The main goal of this work was to study the chemical composition of the lipophilic extractives of bark residues from two *Eucalyptus* species, namely *E. nitens* and *E. grandis x globulus*, widely used in the production of cellulose pulp.

In the outer bark extract of *E. nitens*, triterpenic compounds such as betulinic, betulonic, ursolic and oleanolic acids were identified as the major components. However, in *E. grandis x globulus* the major compound of the outer bark was the methyl-3-hydroxyolean-18-en-28-oate. This compound was identified here for the first time in *Eucalyptus* bark. The inner bark fraction of both species is richer in fatty acids and sterols, being *β*-sitosterol the major component.

From a quantitative point of view, *E. nitens* bark shows to be the best source of triterpenic compounds, with more than 20 g/Kg of outer bark, a higher value than that previously reported for *E. globulus*.

In sum, we can conclude that both *Eucalyptus* bark residues, especially the outer bark fractions, have an enormous potential for the exploitation of these high value compounds with a large range of bioactive properties, making them extremely important and valuable within the biorefinery concept.

5.1 FUTURE WORK

The work carried out may constitute an important point on the characterization and valorization of *Eucalyptus* bark fractions, however, several additional topics raised during the present research should be addressed aiming to enhance the upgrade of these residues:

–The evaluation of the biological activity of methyl-3-hydroxyolean-18-en-28-oate is very important considering its abundance.

-The characterization of the phenolic fraction is also very interesting because of the well-known properties and biological activities of this family of extractives

-The search for more environmental friendly extraction and fractionation methodologies, such as the extraction with supercritical fluids (such as CO_2), is imperative in the context of the biorefinery.

6. REFERENCES

1. Gupta, A.K., Origin of agriculture and domestication of plants and animals linked to early Holocene climate amelioration. *Curr. Sci.*, 2004. **87**(1): 54-59.
2. Centi, G., P. Lanzafame, and S. Perathoner, Analysis of the alternative routes in the catalytic transformation of lignocellulosic materials. *Catal. Today*, 2010. **167**(1): 14-30.
3. Kamm, B., P. Gruber, and M. Kamm, *Biorefineries - Industrial processes and products. Status Quo and Future Directions*. Vol. 1. 2006, Weinheim: WILEY-VCH. p 497.
4. Okkerse C., H.v.B., From fossil to green. *Green Chem.*, 1999: 107-114.
5. Dale, B.E., Did the NPRA mislead the US Senate on ethanol potential? *Biofuels Bioprod. Biorefining*, 2007. **1**(4): 243-244.
6. Ragauskas, A.J., C.K. Williams, B.H. Davison, G. Britovsek, J. Cairney, C.A. Eckert, W.J. Frederick, Jr., J.P. Hallett, D.J. Leak, C.L. Liotta, J.R. Mielenz, R. Murphy, R. Templer, and T. Tschaplinski, The Path Forward for Biofuels and Biomaterials. *Science*, 2006. **311**(5760): 484-489.
7. Demirbas, A., *Biofuels - Green energy and technology*. 2009, London: Springer. p 336.
8. McKendry, P., Energy production from biomass (part 1): overview of biomass. *Bioresour. Technol.*, 2002. **83**(1): 37-46.
9. Rowell, R.M., *Handbook of Wood Chemistry and Wood Composites*. 2005, Florida: CRC Press. p 487.
10. Demirbas, A., Biorefineries: Current activities and future developments. *Energy Conv. Manag.*, 2009. **50**(11): 2782-2801.
11. Fernando, S., S. Adhikari, C. Chandrapal, and N. Murali, Biorefineries: Current Status, Challenges, and Future Direction. *Energy Fuels*, 2006. **20**(4): 1727-1737.
12. Kamm, B. and M. Kamm, Principles of biorefineries. *Appl. Microbiol. Biotechnol.*, 2004. **64**(2): 137-145.
13. Tay, D.H.S., D.K.S. Ng, N.E. Sammons, and M.R. Eden, Fuzzy Optimization Approach for the Synthesis of a Sustainable Integrated Biorefinery. *Ind. Eng. Chem. Res.*, 2011. **50**(3): 1652-1665.
14. Domingues, R.M.A., G.D.A. Sousa, C.S.R. Freire, A.J.D. Silvestre, and C.P. Neto, Eucalyptus globulus biomass residues from pulping industry as a source of high value triterpenic compounds. *Ind. Crop. Prod.*, 2009. **31**(1): 65-70.
15. Freire, C.S.R., A.J.D. Silvestre, and C.P. Neto, Lipophilic extractives in Eucalyptus globulus kraft pulps. Behavior during ECF bleaching. *J. Wood Chem. Technol.*, 2005. **25**(1-2): 67-80.
16. Autoridade Florestal Nacional, *Inventário do IFN 2005| 06*, Ministério da Agricultura do Desenvolvimento Rural e das Pescas, Editor. 2007. p. 70.
17. Boland, D.J., M.I.H. Brooker, G.M. Chippendale, N. Hall, B.P.M. Hyland, R.D. Johnston, D.A. Kleinig, M.W. McDonald, and J.D. Turner, *Forest trees of Australia*. 5th ed. 2006: CSIRO. p 736.
18. Myburg, A.A., A.R. Griffin, R.R. Sederoff, and R.W. Whetten, Comparative genetic linkage maps of Eucalyptus grandis, Eucalyptus globulus and their F-1

hybrid based on a double pseudo-backcross mapping approach. *Theor. Appl. Genet.*, 2003. **107**(6): 1028-1042.
19. Freire, C.S.R., A.J.D. Silvestre, and C.P. Neto, Identification of new hydroxy fatty acids and ferulic acid esters in the wood of Eucalyptus globulus. *Holzforschung*, 2002. **56**(2): 143-149.
20. Downes, G.M., R. Meder, N. Ebdon, H. Bond, R. Evans, K. Joyce, and S. Southerton, Radial variation in cellulose content and Kraft pulp yield in Eucalyptus nitens using near-infrared spectral analysis of air-dry wood surfaces. *J. Near Infrared Spectrosc.* **18**(2): 147-155.
21. Perez-Cruzado, C. and R. Rodriguez-Soalleiro, Improvement in accuracy of aboveground biomass estimation in Eucalyptus nitens plantations: Effect of bole sampling intensity and explanatory variables. *For. Ecol. Manage.* **261**(11): 2016-2028.
22. Demirbas, M.F., Biorefineries for biofuel upgrading: A critical review. *Appl. Energy*, 2009. **86**: S151-S161.
23. Huang, H.J., S. Ramaswamy, U.W. Tschirner, and B.V. Ramarao, A review of separation technologies in current and future biorefineries. *Sep. Purif. Technol.*, 2008. **62**(1): 1-21.
24. Yang, P.F., H. Kobayashi, and A. Fukuoka, Recent Developments in the Catalytic Conversion of Cellulose into Valuable Chemicals. *Chin. J. Catal.*, 2011. **32**(5): 716-722.
25. Bledzki, A.K. and J. Gassan, Composites reinforced with cellulose based fibres. *Prog. Polym. Sci.*, 1999. **24**(2): 221-274.
26. Freire, C.S.R., A.J.D. Silvestre, C.P. Neto, and J.A.S. Cavaleiro, Lipophilic extractives of the inner and outer barks of Eucalyptus globulus. *Holzforschung*, 2002. **56**(4): 372-379.
27. Liu, J., Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.*, 1995. **49**(2): 57-68.
28. Gupta, M.B., R. Nath, G.P. Gupta, and K.P. Bhargava, Anti-ulcer activity of some plant triperpenoids. *Indian J. Med. Res.*, 1981. **73**(APR): 649-652.
29. Kondo, M., S.L. MacKinnon, C.C. Craft, M.D. Matchett, R.A.R. Hurta, and C.C. Neto, Ursolic acid and its esters: occurrence in cranberries and other Vaccinium fruit and effects on matrix metalloproteinase activity in DU145 prostate tumor cells. *J. Sci. Food Agric.*, 2011. **91**(5): 789-796.
30. Limami, Y., A. Pinon, D.Y. Leger, Y. Mousseau, J. Cook-Moreau, J.L. Beneytout, C. Delage, B. Liagre, and A. Simon, HT-29 colorectal cancer cells undergoing apoptosis overexpress COX-2 to delay ursolic acid-induced cell death. *Biochimie*, 2011. **93**(4): 749-757.
31. Bishayee, A., S. Ahmed, N. Brankov, and M. Perloff, Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Front. Biosci.*, 2011. **16**: 980-996.
32. Aggarwal, B.B. and S. Shishodia, Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.*, 2006. **71**(10): 1397-1421.
33. Alakurtti, S., T. Makela, S. Koskimies, and J. Yli-Kauhaluoma, Pharmacological properties of the ubiquitous natural product betulin. *Eur. J. Pharm. Sci.*, 2006. **29**(1): 1-13.
34. Santos, R.C., J.A.R. Salvador, R. Cortes, G. Pachon, S. Marin, and M. Cascante, New betulinic acid derivatives induce potent and selective antiproliferative

activity through cell cycle arrest at the S phase and caspase dependent apoptosis in human cancer cells. *Biochimie*, 2011. **93**(6): 1065-1075.

35. Qian, K.D., R.Y. Kuo, C.H. Chen, L. Huang, S.L. Morris-Natschke, and K.H. Lee, Anti-AIDS Agents 81. Design, Synthesis, and Structure-Activity Relationship Study of Betulinic Acid and Moronic Acid Derivatives as Potent HIV Maturation Inhibitors. *J. Med. Chem.*, 2010. **53**(8): 3133-3141.
36. Pegel, K.H., The importance of sitosterol and sitosterolin in human and animal nutrition. *S. Afr. J. Sci.*, 1997. **93**(6): 263-268.
37. Donald, P.R., J.H. Lamprecht, M. Freestone, C.F. Albrecht, P.J.D. Bouic, D. Kotze, and P.P. van Jaarsveld, A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.*, 1997. **1**(6): 518-522.
38. Romer, S. and N. Garti, The activity and absorption relationship of cholesterol and phytosterols. *Colloid Surf. A-Physicochem. Eng. Asp.*, 2006. **282**: 435-456.
39. Alappat, L., M. Valerio, and A.B. Awad, Effect of vitamin D and beta-sitosterol on immune function of macrophages. *Int. Immunopharmacol.*, 2010. **10**(11): 1390-1396.
40. Villaflores, O.B., A.P.G. Macabeo, D. Gehle, K. Krohn, S.G. Franzblau, and A.M. Aguinaldo, Phytoconstituents from Alpinia purpurata and their in vitro inhibitory activity against Mycobacterium tuberculosis. *Pharmacogn. Mag.*, 2010. **6**(24): 339-344.
41. Chen, J., X.-s. Dong, and X.-g. Guo, Inhibitory effect of resveratrol on the growth of human colon cancer ls174t cells and its subcutaneously transplanted tumor in nude mice and the mechanism of action. *Zhonghua Zhong Liu Za Zhi*, 2009. **31**(1): 15-19.
42. Csiszar, A., *Anti-inflammatory effects of resveratrol: possible role in prevention of age-related cardiovascular disease*. Resveratrol and Health. Vol. 1215. 2011. p 117-122.
43. He, X., Y. Wang, J.H. Zhu, M. Orloff, and C. Eng, Resveratrol enhances the anti-tumor activity of the mTOR inhibitor rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced AKT signaling. *Cancer Lett.*, 2011. **301**(2): 168-176.
44. Frame, A.A., M.A. Kluge, S.M. Shenouda, M.L. Hartman, M. Holbrook, M.A. Duess, A. Levit, B.H. Kim, T.L. Caiano, L. Fraser, C.E. Tabit, E. Tampakakis, N.M. Hamburg, and J.A. Vita, The effects of resveratrol on endothelial function in patients with Type 2 diabetes mellitus. *Vasc. Med.*, 2011. **16**(3): 21.
45. Luo, H.T., B.H. Jiang, S.M. King, and Y.C. Chen, Inhibition of Cell Growth and VEGF Expression in Ovarian Cancer Cells by Flavonoids. *Nutr. Cancer*, 2008. **60**(6): 800-809.
46. Shen, S.C., C.H. Ko, S.W. Tseng, S.H. Tsai, and Y.C. Chen, Structurally related antitumor effects of flavanones in vitro and in vivo: involvement of caspase 3 activation, p21 gene expression, and reactive oxygen species production. *Toxicol. Appl. Pharmacol.*, 2004. **197**(2): 84-95.
47. Peuhu, E., A. Rivero-Muller, H. Stykki, E. Torvaldson, T. Holmbom, P. Eklund, M. Unkila, R. Sjoholm, and J.E. Eriksson, Inhibition of Akt signaling by the lignan matairesinol sensitizes prostate cancer cells to TRAIL-induced apoptosis. *Oncogene*, 2010. **29**(6): 898-908.
48. Peuhu, E., A. Rivero-Muller, H. Stykki, P. Tuominen, E. Torvaldson, M. Unkila, and J.E. Eriksson, Sensitization of prostate cancer cells to trail-mediated

apoptosis by the tumor-suppressing matairesinol lignan. *Advances in Tnf Family Research*, 2011. **691**: 798-798.
49. Sjöström, E., *Wood chemistry-Fundamentals and applications 2nd ed.* 1993, San Diego: Academic Press Inc. p 293.
50. Shebani, A.N., A.J. van Reenen, and M. Meincken, The effect of wood extractives on the thermal stability of different wood species. *Thermochim. Acta*, 2008. **471**(1-2): 43-50.
51. Ekman, R. and B. Holmbom, *Pitch control, Wood resin and Deresination*, E. Ernst L. Black and Lawrence H. Allen. 2000, Atlanta: Tappi press.
52. Gutierrez, A., J.C. del Rio, F.J. Gonzalez-Vila, and F. Martin, Chemical composition of lipophilic extractives from Eucalyptus globulus Labill. wood. *Holzforschung*, 1999. **53**(5): 481-486.
53. Swan, B. and I.S. Akerblom, Wood extractives from *Eucalyptus globulus* labill. *Sven. Papperstidn.*, 1967. **70**(7): 239-&.
54. Fengel, D. and G. Wegener, *Wood. Chemistry, ultrastructure, reactions*. 1984, Berlin: Walter de Gruyter.
55. Alzogaray, R.A., A. Lucia, E.N. Zerba, and H.M. Masuh, Insecticidal activity of essential oils from eleven Eucalyptus spp. and two hybrids: lethal and sublethal effects of their major components on Blattella germanica. *J. Econ. Entomol.* **104**(2): 595-600.
56. Rowe, J.W., *Natural products of woody plants I*. 1989, Berlin: Springer-Verlag.
57. Xu, R., G.C. Fazio, and S.P.T. Matsuda, On the origins of triterpenoid skeletal diversity. *Phytochemistry*, 2004. **65**(3): 261-291.
58. J. Mann, R.S.D., J.B. Hobbs, D.V. Banthorpe and and J.B. Harborne, *Phenolics*, in *Natural Products: Their Chemistry and Biological Significance*. 1994, Longman Scientific & Technical: United Kingdom. 361-388.
59. Hillis, W.E., Contribution of polyphenolic wood extractives to pulp colour. *Appita J.*, 1969. **23**(2): 89-101.
60. Jung, H.G. and D.A. Deetz, *Forage cell wall structure and digestibilty*. 1993, Madison: ASA-CSSASSSA.
61. Santos, S.A.O., C.S.R. Freire, M.R.M. Domingues, A.J.D. Silvestre, and C.P. Neto, Characterization of Phenolic Components in Polar Extracts of Eucalyptus globulus Labill. Bark by High-Performance Liquid Chromatography-Mass Spectrometry. *J. Agric. Food Chem.*, 2011: null-null.
62. Tapiero, H., K.D. Tew, G.N. Ba, and G. Mathe, Polyphenols: do they play a role in the prevention of human pathologies? *Biomed. Pharmacother.*, 2002. **56**(4): 200-207.
63. Stewart, D., G.W. Robertson, and I.M. Morrison, Phenolic acids dimers in the cell walls of barley. *Biol. Mass Spectrom.*, 1994. **23**(2): 71-74.
64. Hsieh, T.-C., C. Wong, D. John Bennett, and J.M. Wu, Regulation of p53 and cell proliferation by resveratrol and its derivatives in breast cancer cells: An in silico and biochemical approach targeting integrin alphavbeta3. *International journal of cancer. Journal international du cancer*, 2011. **129**(11): 2732-43.
65. Hillis, W.E., J.H. Hart, and Y. Yazaki, Polyphenols of *Eucalyptus-sideroxylon wood. Phytochemistry*, 1974. **13**(8): 1591-1595.
66. West, M.E. and L.J. Mauer, Development of an integrated approach for the stability testing of flavonoids and ascorbic acid in powders. *Food Chem.*, 2011. **129**(1): 51-58.

67. Jordheim, M., F. Mage, and O.M. Andersen, Anthocyanins in berries of Ribes including gooseberry cultivars with a high content of acylated pigments. *J. Agric. Food Chem.*, 2007. **55**(14): 5529-5535.
68. Monagas, M., I. Garrido, B. Bartolome, and C. Gomez-Cordoves, Chemical characterization of commercial dietary ingredients from Vitis vinifera L. *Anal. Chim. Acta*, 2006. **563**(1-2): 401-410.
69. Conde, E., E. Cadahia, R. DiezBarra, and M.C. GarciaVallejo, Polyphenolic composition of bark extracts from Eucalyptus camaldulensis, E-globulus and E-rudis. *Holz Als Roh-und Werkst.*, 1996. **54**(3): 175-181.
70. Conde, E., E. Cadahia, M. Garciavallejo, and F. Tomasbarberan, Low molecular weight poliphenols in wood and bark of Eucalyptus globulus. *Wood Fiber Sci.*, 1995. **27**(4): 379-383.
71. Ghosh, P. and G.P. Fenner, Improved method for gas chromatographic analysis of genistein and daidzein from soybean (Glycine max) seeds. *J. Agric. Food Chem.*, 1999. **47**(9): 3455-3456.
72. Ovenden, S.P.B., J. Yu, S.S. Wan, G. Sberna, R.M. Tait, D. Rhodes, S. Cox, J. Coates, N.G. Walsh, and B.M. Meurer-Grimes, Globoidnan A: a lignan from Eucalyptus globoidea inhibits HIV integrase. *Phytochemistry*, 2004. **65**(24): 3255-3259.
73. Saarinen, N.M., A. Warri, M. Airio, A. Smeds, and S. Makela, Role of dietary lignans in the reduction of breast cancer risk. *Mol. Nutr. Food Res.*, 2007. **51**(7): 857-866.
74. Pereira, S.I., C.S.R. Freire, C.P. Neto, A.J.D. Silvestre, and A.M.S. Silva, Chemical composition of the epicuticular wax from the fruits of Eucalyptus globulus. *Phytochem. Anal.*, 2005. **16**(5): 364-369.
75. Yun, B.S., I.K. Lee, J.P. Kim, S.H. Chung, G.S. Shim, and I.D. Yoo, Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of Eucalyptus globulus. *Arch. Pharm. Res.*, 2000. **23**(2): 147-150.
76. Barry, K.M., N.W. Davies, and C.L. Mohammed, Identification of hydrolysable tannins in the reaction zone of Eucalyptus nitens wood by high performance liquid chromatography-electrospray ionisation mass spectrometry. *Phytochem. Anal.*, 2001. **12**(2): 120-127.
77. Hou, A.J., Y.Z. Liu, H. Yang, Z.W. Lin, and H.D. Sun, Hydrolyzable tannins and related polyphenols from Eucalyptus globulus. *J. Asian Nat. Prod. Res.*, 2000. **2**(3): 205-212.
78. Cadahia, E., E. Conde, B.F. deSimon, and M.C. GarciaVallejo, Tannin composition of Eucalyptus camaldulensis, E-globulus and E-rudis .2. Bark. *Holzforschung*, 1997. **51**(2): 125-129.
79. Cadahia, E., E. Conde, M.C. GarciaVallejo, and B.F. deSimon, Tannin composition of Eucalyptus camaldulensis, E-globulus and E-rudis .1. Wood. *Holzforschung*, 1997. **51**(2): 119-124.
80. Sithole, B.B., Modern methods for the analysis of extractives from wood and pulp - A Review. *Appita J.*, 1992. **45**(4): 260-264.
81. Villaverde, J.J., A. De Vega, P. Ligero, C.S.R. Freire, C.P. Neto, and A.J.D. Silvestre, Miscanthus x giganteus Bark Organosolv Fractionation: Fate of Lipophilic Components and Formation of Valuable Phenolic Byproducts. *J. Agric. Food Chem.*, 2010. **58**(14): 8279-8285.
82. Villaverde, J.J., R.M.A. Domingues, C.S.R. Freire, A.J.D. Silvestre, C.P. Neto, P. Ligero, and A. Vega, Miscanthus x giganteus Extractives: A Source of

Valuable Phenolic Compounds and Sterols. *J. Agric. Food Chem.*, 2009. **57**(9): 3626-3631.
83. Kitson, F.G., B.S. Larsen, and C.N. McEwen, *Gas chromatography and mass spectrometry: a practical guide*. Academic Press Inc. 1996, San Diego.
84. Villaverde, J.J., S.A.O. Santos, M.M.Q. Simoes, C.P. Neto, M.R.M. Domingues, and A.J.D. Silvestre, Analysis of linoleic acid hydroperoxides generated by biomimetic and enzymatic systems through an integrated methodology. *Ind. Crop. Prod.*, 2011. **34**(3): 1474-1481.
85. Santos, S.A.O., P.C.R.O. Pinto, A.J.D. Silvestre, and C.P. Neto, Chemical composition and antioxidant activity of phenolic extracts of cork from Quercus suber L. *Ind. Crop. Prod.*, 2010. **31**(3): 521-526.
86. Singleton, V.L. and J.A. Rossi, Jr., Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.*, 1965. **16**(3): 144-158.
87. Sharma, O.P. and T.K. Bhat, DPPH antioxidant assay revisited. *Food Chem.*, 2009. **113**(4): 1202-1205.
88. Wongkittipong, R., L. Prat, S. Damronglerd, and C. Gourdon, Solid-liquid extraction of andrographolide from plants-experimental study, kinetic reaction and model. *Sep. Purif. Technol.*, 2004. **40**(2): 147-154.
89. Domingues, R.M.A., G.D.A. Sousa, C.M. Silva, C.S.R. Freire, A.J.D. Silvestre, and C. Pascoal Neto, High value triterpenic compounds from the outer barks of several Eucalyptus species cultivated in Brazil and in Portugal. *Ind. Crop. Prod.*, 2011. **33**(1): 158-164.
90. Domingues, R.M.A., D.J.S. Patinha, G.D.A. Sousa, J.J. Villaverde, C.M. Silva, C.S.R. Freire, A.J.D. Silvestre, and C.P. Neto, Eucalyptus biomass residues from agro-forest and pulping industries as sources of high-value triterpenic compounds. *Cell Chem. Technol.*, 2011. **45**(7-8): 475-481.
91. Assimopoulou, A.N. and V.P. Papageorgiou, GC-MS analysis of penta- and tetra-cyclic triterpenes from resins of Pistacia species. Part II. Pistacia terebinthus var. Chia. *Biomed. Chromatogr.*, 2005. **19**(8): 586-605.
92. Mathe, C., G. Culioli, P. Archier, and C. Vieillescazes, Characterization of archaeological frankincense by gas chromatography-mass spectrometry. *J. Chromatogr. A*, 2004. **1023**(2): 277-285.
93. Pelillo, M., G. Iafelice, E. Marcon, and M.F. Caboni, Identification of plant sterols in hexaploid and tetraploid wheats using gas chromatography with mass spectrometry. *Rapid Commun. Mass Spectrom.*, 2003. **17**(20): 2245-2252.
94. Merrit, C.J. and C.N. McEwen, *Mass spectrometry - Part B*. Marcell Dekker, Inc. 1980, New York.
95. Andrasi, N., A. Helenkar, G. Zaray, A. Vasanits, and I. Molnar-Perl, Derivatization and fragmentation pattern analysis of natural and synthetic steroids, as their trimethylsilyl (oxime) ether derivatives by gas chromatography mass spectrometry: Analysis of dissolved steroids in wastewater samples. *J. Chromatogr. A*, 2011. **1218**(14): 1878-1890.
96. Evershed, R.P., *Lipid analysis - A pratical aproach*, R.J. Hamilton and S. Hamilton. 1992, Oxford: IRL Press.
97. Murphy, R.C., *Handbook of lipid research*, F. Snyder. 1993, New York: Plenum Press.
98. Burnouf-Radosevich, M., N.E. Delfel, and R. England, Gas chromatography - mass spectrometry of oleanane-type and ursane-type triterpenes - Application to chenopodium-quinoa triterpenes. *Phytochemistry*, 1985. **24**(9): 2063-2066.

99. Assimopoulou, A.N. and V.P. Papageorgiou, GC-MS analysis of penta- and tetra-cyclic triterpenes from resins of Pistacia species. Part I. Pistacia lentiscus var. Chia. *Biomed. Chromatogr.*, 2005. **19**(4): 285-311.
100. Razborsek, M.I., D.B. Voncina, V. Dolecek, and E. Voncina, Determination of major phenolic acids, phenolic diterpenes and triterpenes in rosemary (Rosmarinus officinalis L.) by gas chromatography and mass spectrometry. *Acta Chim. Slov.*, 2007. **54**(1): 60-67.
101. Hart, N.K. and Lamberto.Ja, Morolic acid (3-Hydroxyolean-18-en-28-oic acid) from bark of *Eucalyptus papuana* F Muell. *Aust. J. Chem.*, 1965. **18**(1): 115-&.
102. Cannon, J.R., B.W. Metcalf, C.L. Raston, and A.H. White, Isolation o 3Beta-hydroxyolean-18-en-28-oic acid (morolic acid) from *Eucalyptus grossa* and the crysta-structure of methyl morolate acetate *Aust. J. Chem.*, 1981. **34**(5): 1134-1144.
103. Budzikiewicz, H., J.M. Wilson, and C. Djerassi, Mass spectrometry in structural and stereochemical problems .32. Pentacyclic triterpenes. *J. Am. Chem. Soc.*, 1963. **85**(22): 3688.
104. Seebacher, W., N. Simic, R. Weis, R. Saf, and O. Kunert, Complete assignments of H-1 and C-13 NMR resonances of oleanolic acid, 18 alpha-oleanolic acid, ursolic acid and their 11-oxo derivatives. *Magn. Reson. Chem.*, 2003. **41**(8): 636-638.
105. Nunez, M.J., C.P. Reyes, I.A. Jimenez, L. Moujir, and I.L. Bazzocchi, Lupane triterpenoids from Maytenus species. *J. Nat. Prod.*, 2005. **68**(7): 1018-1021.
106. Gutierrez, R.M.P., Pentacyclic triterpenes from Cirsium pascuarense. *J. Chil. Chem. Soc.*, 2005. **50**(3): 587-589.
107. Vázquez, G., E. Fontenla, J. Santos, M.S. Freire, J. González-Álvarez, and G. Antorrena, Antioxidant activity and phenolic content of chestnut (Castanea sativa) shell and eucalyptus (Eucalyptus globulus) bark extracts. *Ind. Crop. Prod.*, 2008. **28**(3): 279-285.